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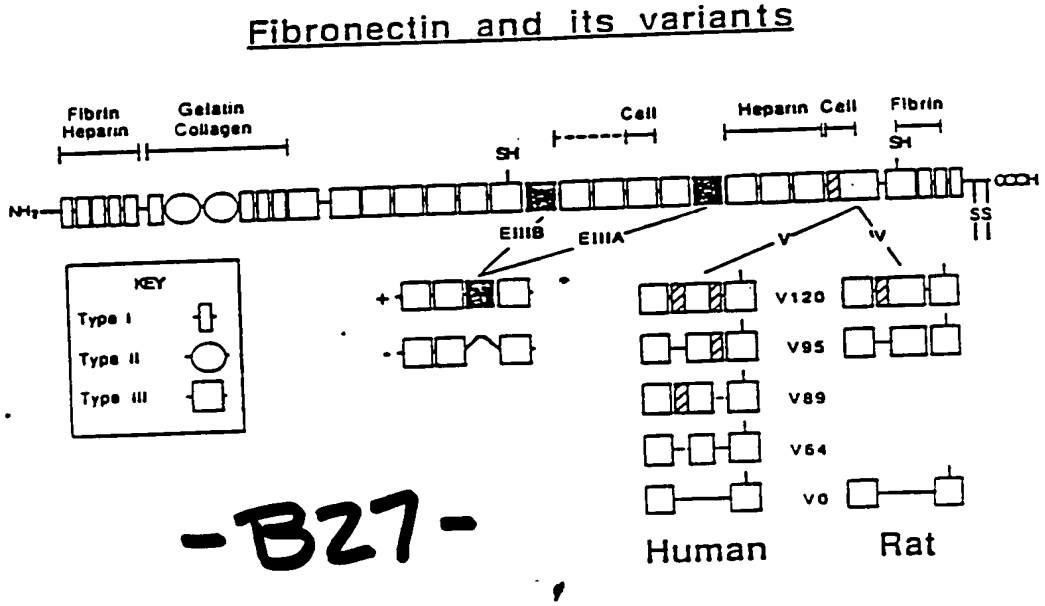
(54) Title: EXPRESSION OF RECOMBINANT FIBRONECTIN IN GENETICALLY ENGINEERED CELLS

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(57) Abstract

A method of producing homogeneous cellular fibronectin of mammalian origin, which is a homodimer, as well as recom nant cellular fibronectin. They can be produced having all or a portion of regions B, A, V or combinations of these. Specifical homodimers and heterodimers can be produced having the carboxy terminal 25 amino acids of region V deleted. The recom nant cellular fibronectin is useful in any application in which naturally-occurring fibronectin can be used.

They are joined by disulfide bonds very near their C-termini.

Each subunit is made up of a series of tightly-folded globular domains, each of which is specialized for binding to other molecules or to cells. Hynes, R., Ann. Rev. Cell. Biol., 1:67-90 (1985). They are composed of a series of homologous repeating units of three types: Type I and II homologies, which are disulfide-bonded loops each 45-50 amino acids long, and Type III homologies, which are 90 amino acids long and lack disulfide bonds. Patel, R.S. et al., The EMBO Journal, 6:2565-2572 (1987). Hynes, Ann. Rev. Cell. Biol., 1:67-90 (1985). Recombinant DNA analyses have shown that although different subunits differ in parts of their primary sequence, they arise from a single gene and are identical over much of their sequence. Kornblihtt et al., Proceedings of the National Academy of Sciences, USA, 80:3218-3222 (1983). More than 90% of the sequence of fibronectin is made up of repeats of these three homologies. However, studies have indicated that fibronectins from different sources, e.g., fibroblasts (cellular) and plasma, are not identical. For example, plasma fibronectin contains subunits of two different mobilities on SDS - polyacrylamide gels and fibronectin from fibroblasts (cellular fibronectin) shows a different subunit pattern. Paul, J.I. et al., J. Biol. Chem., 261:12258-12265 (1986).

Recently, it has been confirmed that two Type III repeats, designated EIIIA and EIIIB, are alternatively spliced and are each encoded by a single exon. Both EIIIA and EIIIB are always omitted by liver cells,

# EXPRESSION OF RECOMBINANT FIBRONECTIN IN GENETICALLY ENGINEERED CELLS

## Background

The interactions of cells with one another and with extracellular materials (e.g., matrices, solid surfaces) are of vital importance for cell function. These interactions have major effects on the proliferation, differentiation, and organization of cells. These interactions are often mediated by a class of high molecular weight glycoproteins that are involved both in these interactions and in the actual structure of extracellular matrices. One important glycoprotein of this class is fibronectin. Hynes, R.O., Ann. Rev. Cell. Biol., 1:67-90 (1985); Hynes, R.O., Scientific American, 254:42-51 (1986); Hynes, R.O. and K.M. Yamada, J. Cell. Biol., 95:369-377 (1982).

Fibronectins are high molecular weight glycoproteins involved in cell adhesion, morphology and migration. Fibronectin has been shown to consist of a dimer of two subunits, each about 250 kilodaltons in size. The two subunits, which are similar, but not necessarily identical, each fold into an elongated and flexible arm.

For example, fibronectin is thought to play an important role in the cell processes involved in tissue repair, particularly wound healing. The ability to repair damaged tissue, wound healing, represents an important response to injury that is common to all complex organisms. Just as in embryonic development, this process involves cell proliferation, migration and drfferentiation of a number of different cell types. Fibronectin promotes cell migration in culture and is present in the embryo associated with many different cell migrations. In addition, antibodies to fibronectin or to cell surface receptors of the integrin glycoprotein family can block migration when injected into the intact embryo.

Fibronectin is expressed at high levels in healing wounds. It is derived from two sources: plasma fibronectin, which is present in the exudate from damaged blood vessels, and cellular fibronectin, which is synthesized locally in the wound tissue. Fibronectin appears to be involved in the migration <u>in vitro</u> of four major cell types that migrate into the area of the wound. Fibroblasts and epithelial cells are stimulated to migrate by fibronectin.

Presently, only plasma fibronectin is available (e.g., for therapeutic uses) in quantity. However, it is a mixture of variants and is impure and, thus, its use in treatment may be of limited value, particularly in those circumstances in which a pure form or a combination of selected variants, rather than a mixture of variants, would be more effective.

although both can be included by other cell types and all possible combinations occur. Because both EIIIA and EIIIB are omitted by hepatocytes, neither repeat occurs in plasma fibronectin. It is interesting to note that a third region, designated V, is also alternatively spliced. Unlike EIIIA and EIIIB, however, it is alternatively spliced both in fibroblasts and hepatocytes. Schwarzbauer, J.E. et al., The EMBO Journal, 6:2573-2580 (1987). Thus, the V region can be present in both plasma fibronectin and fibroblast or cellular fibronectin. For cellular fibronectin, therefore, there are eight possible combinations or variants of these three alternatively spliced regions:

| Combination | Designated herein as |
|---|---|
| $B^-A^-V^-$ | O |
| $B^+A^-V^-$ | B |
| $B^-A^+V^-$ | A |
| $B^-A^-V^+$ | V |
| $B^+A^-V^+$ | BV |
| $B^-A^+V^+$ | AV |
| $B^+A^+V^-$ | BA |
| $B^+A^+V^+$ | BAV |

For plasma fibronectin, there can be no variants in which A and B are present.

Fibronectins play an important role in many biological systems. They have been shown to be involved in cell adhesion and migration, cell morphology, hemostasis, thrombosis and oncogenic transformation. Hynes, R.O. and K.M. Yamada, J. Cell. Biol., 95:369-377 (1982) and Hynes, R.O. Scientific American, 254:42-51 (1986).

For example, fibronectin is thought to play an important role in the cell processes involved in tissue repair, particularly wound healing. The ability to repair damaged tissue, wound healing, represents an important response to injury that is common to all complex organisms. Just as in embryonic development, this process involves cell proliferation, migration and drfferentiation of a number of different cell types. Fibronectin promotes cell migration in culture and is present in the embryo associated with many different cell migrations. In addition, antibodies to fibronectin or to cell surface receptors of the integrin glycoprotein family can block migration when injected into the intact embryo.

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Presently, only plasma fibronectin is available (e.g., for therapeutic uses) in quantity. However, it is a mixture of variants and is impure and, thus, its use in treatment may be of limited value, particularly in those circumstances in which a pure form or a combination of selected variants, rather than a mixture of variants, would be more effective.

Summary of the Invention

The present invention relates to a method of producing cellular fibronectin of mammalian origin through the use of genetic engineering techniques, as well as to cellular fibronectins produced by the method. Until now, it has not been possible to produce essentially pure cellular fibronectin. The fibronectins of the present invention, however, are homogeneous cellular fibronectins which include, as desired, region B, region A or both regions, as well as the V region, if desired. Recombinant fibronectins having portions of these regions can also be produced. Thus, the fibronectins of the present invention differ from presently-available fibronectins in that the subject fibronectins are recombinant homogeneous cellular fibronectins which include region B and/or region A, alone or in combination.

In one embodiment of the method of the present invention, a recombinant full length cDNA encoding cellular fibronectin is introduced into an appropriate host cell by means of a recombinant retrovirus (or other suitable vector). The full length cDNA is expressed in the host cell, resulting in production of full length cellular fibronectin. The eight possible variants of full length rat cellular fibronectin (i.e., those including some, none or all of the three alternative splice domains designated A, B and V) have been expressed in this way, characterized, and shown, using art-recognized methods, to be biologically functional. Characterization of the recombinant cellular fibronectin produced as described herein has demonstrated that the method results in production of homogeneous populations of homodimers

(rather than the mixtures of heterodimers which naturally occur).

Brief Description of the Drawings

Figure 1 is a schematic representation of fibronectin and its variants.

Figure 2 is a schematic representation of the method of the present invention, by which recombinant full length fibronectin is produced.

Figure 3 shows the entire nucleic acid sequence encoding recombinant full-length rat fibronectin.

Figures 4a and 4b demonstrate expression of different variants of fibronectin in WEHI231 cells, which are lymphocytes which do not themselves produce fibronectin. Immunoprecipitation, using a polyclonal antibody (R61.1) which recognizes total fibronectin, was carried out, followed by reduced and nonreduced gel electrophoresis. WEHI231 cells infected with a recombinant vector containing one of the following were analyzed: pLJ, control (no fibronectin variant); O, $B^-A^-V^-$; B, $B^+A^-V^-$; A, $B^-A^+V^-$; V, $B^-A^-V^+$. Results of reduced gel electrophoresis demonstrate the presence of the fibronectin monomer and results of nonreduced gel electrophoresis demonstrate dimerization of fibronectin.

Figure 5 is a graphic representation of spreading or adhesion of melanoma cells (B16F10) on various variants and concentrations of recombinant fibronectin produced in WEHI231 cells. O, B, A and V indicate the fibronectin variants used (see Figure 4 for variant type).

Figures 6a, b and c demonstrate the ability of recombinant fibronectin variant A ($B^-A^+V^-$) produced in WEHI231 cells to promote reversion of Nil.8-HSV morphology to a normal morphology.

Figures 7a-f demonstrate the results of assessment of extracellular matrices of NIH 3T3 cells and incorporation of recombinant fibronectin variants B ($B^+A^-V^-$) and V ($B^-A^-V^+$) produced in WEHI231 cells into the matrices at three concentrations: -, no added fibronectin; 30 ug/ml.; 90 ug/ml.

Figure 8 is a schematic representation of the rat fibronectin structure which is composed of three repeating peptide units termed Type I, II and III, which are shown by boxes. Two alternatively spliced type III repeats, EIIIB and EIIIA, are represented by the filled squares and are indicated above the diagram. The V region is marked by the shaded box and the V25 segment is indicated with stripes, and its amino acid sequence is given below. Domains that interact with fibrin, collagen, and heparin are illustrated above. The sites that interact with cell surfaces are also shown.

Figure 9 shows the peptide inhibition of WEHI231 cell spreading on the V form of FN. The average scores of three independent experiments for each peptide are shown with their standard deviations (A). The peptide sequences are illustrated in (B). O represents the score in the absence of any peptide competitor. RGD and RGE represent peptides GRGDSP and GRGESP, respectively.

## Detailed Description of the Invention

The present invention provides a method of producing recombinant cellular fibronectin which is essentially

full length (recombinant cellular fibronectin) and of producing modified, essentially full-length recombinant cellular fibronectin. cDNA encoding recombinant cellular fibronectin has been expressed in NIH 3T3 and WEHI231 cells and tested on several cell types (CHO, Rat-1, BHK, B16 melanoma) which are standard cell types used to assay fibronectins. It has been shown, using art-recognized methods, to be produced in the form of essentially pure homogeneous homodimers and to be biologically active.

Recombinant Cellular Fibronectin

The following is a description of construction of cDNA encoding cellular fibronectin variants and of vectors useful for introduction of the cDNA into appropriate host cells, expression of the encoded cellular fibronectin in host cells containing the cDNA and characterization of the recombinant cellular fibronectin produced in this manner. Although the following describes cellular fibronectin of rat origin, it is to be understood that the same procedures can be used with cDNA encoding cellular fibronectin from other sources (e.g., human) to produce the encoded recombinant fibronectins.

Fibronectin and its variants are represented schematically in Figure 1. As shown, fibronectin includes regions which bind to various proteins or other substances (e.g., fibrin, heparin, collagen, gelatin), as well as three regions, described above, designated EIIIB, EIIIA and V. Regions EIIIB and EIIIA can be present in cellular fibronectin, but not in plasma fibronectin. Region V can be present in variants of both cellular and plasma fibronectin. As described above, there are eight

possible combinations of these three regions (eight variants) in the case of full length cellular fibronectin. cDNA encoding each of these eight variants has been produced by combining or joining cDNA fragments, each of which encodes a portion or segment of cellular fibronectin. See, Patel, R.S. et al., The EMBO J., 6:2565-2572 (1987); Schwarzbauer, J.E. et al., The EMBO J., 6:2573-2580 (1987).

The full length cDNA was introduced into appropriate cells, in which it was expressed and subsequently secreted, in the following manner, which is represented schematically in Figure 2. Figure 3 shows the entire nucleic acid sequence encoding recombinant full-length rat fibronectin. Recombinant full length cDNA encoding fibronectin was introduced into the pLJ vector, using known techniques. See, Schwarzbauer, J.E. et al., Proc. of the Natl. Acad. of Sci., USA, 84:754-758 (1987). Schwarzbauer has also modified the vector by removal of a splice site. This modifed vector is called pLJ. The characteristics of pLJ have been described in Korman, A.J. et al., Proc. of the Natl. Acad. of Sci., USA, 84:2150 (1987). This vector is capable of expressing both the gene of interest and a dominant selectable marker, such as the neo gene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5' LTR, while the Neo gene is placed distal to an internal promoter (from SV40) which is located 3' of the cloning site. Transcription from pLJ is initiated at two sites: 1) the 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the neo gene.

The diagram at the top of Figure 2 is a representation of pLJ and the additional sequences inserted into it (e.g., full length cDNA encoding cellular fibronectin and a neomycin resistance-encoding gene, NEO-R), resulting in production of a recombinant retrovirus designated pLJ-FN. The NEO-R gene product confers resistance to the antibiotic, G418, in mammalian cells and can be used to select cells containing the recombinant vector. The resulting plasmid (pLJ-FN) was introduced into a packaging cell (e.g., Psi 2 cells), in which the cDNA was transcribed and the resulting fibronectin mRNA and NEO-R mRNA incorporated or packaged into viral particles which subsequently bud out of the cells.

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all virion proteins. Mulligan and co-workers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant stably integrated into the chromosome. Mulligan, R.C., In: Experimental Manipulation of Gene Expression, M. Inouye (ed.), 155-173 (1983); Mann, R. et al., Cell, 33:153-159 (1983); Cone, R.D. and R.C. Mulligan, Proc. of the Natl. Acad. of Sci., USA, 81:6349-6353 (1984).

The Psi 2 cell line described by Mulligan and co-workers was created by transfecting NIH 3T3 fibroblasts with pMOV-Psi$^-$, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-Psi$^-$ expresses all the viral gene products but lacks the Psi

sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi$^-$ expresses an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi$^-$am line, which are Psi-2-like packaging cell lines. These Psi$^-$am cell lines contain a modified pMOV-Psi$^-$genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology, 19:19-25 (1976). As a result, they are useful for production of recombinant virus with a broad mammalian host range, amphotropic host range. The retrovirus used to make the Psi-am cell line has an amphotropic host range and can be used to infect human cells. If the recombinant genome has the Psi packaging sequence, the Psi-am cell line is capable of packaging recombinant retroviral genomes into infectious retroviral particles. Cone, R. and R. Mulligan, Proc. of the Natl. Acad. Sci., USA, 81:6349-6353 (1984).

The retroviral genome has been modified by Cone and Mulligan for use as a vector capable of introducing new genes into cells. The gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene serves as a dominant selectable marker. The retroviral sequence which remains part of the recombinant genome includes the LTRs, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984). In this instance, full-length fibronectin cDNA was constructed as described and inserted into the vector, as indicated in Figure 2.

The resulting recombinant retrovirus (pLJ-FN) was used to infect appropriate recipient/host cells (e.g., NIH 3T3, WEHI231). In these cells, reverse transcription of the viral RNA results in generation of a DNA copy, which integrated into the host cell genome. Infected cells, which were G418 resistant, synthesized the encoded recombinant fibronectin variant, which was subsequently secreted into the culture medium.

Each of the eight fibronectin variants in which EIIIB, EIIIA and/or V can be present was expressed in at least one of the following two types of mammalian cell: NIH 3T3 and WEHI231. Methods for making the variants are described in detail in the Exemplification. WEHI231 cells expressing the V fibronectin variant ($B^-A^-V^+$) have been deposited, according to the terms of the Budapest Treaty, at the American Type Culture Collection (Rockville, MD) under Accession No. CRL10019. The resulting recombinant fibronectin was tested on several cell types (e.g., CHO, Rat-1, BHK, B16 melanoma cells) which are, as mentioned previously, standard cell types used to assay fibronectins. The resulting recombinant fibronectins were characterized and shown to be homodimers, rather than the heterodimers produced naturally. They have been shown, as described below, to be biologically functional. Naturally-occurring fibronectin is known to bind anti fibronectin antibodies, gelatin, and heparin; to promote adhesion of several different cell types, cytoskeletal assembly and cell migration; and to participate in reversion of tumor cells (transformed cells) to normal morphology. As described below, recombinant fibronectin produced as described herein has been shown to have these same capabilities.

Expression of recombinant fibronectin (BAV) in NIH 3T3 cells was clearly demonstrated and the results confirmed by Northern blot analyses. Two transcripts were detected from three individual clones infected with retrovirus containing the BAV form FN gene when the $neo^r$ gene was used as probe. The 11.6- and 3.1-kb messages which were observed corresponded with the sizes expected for the full-length genomic transcript from the viral LTR and subgenomic RNA from the SV-40 promoter. No band was detected in RNA isolated from parental 3T3 cells. RNA isolated from cells containing pLJ vector alone included the 3.1-kb subgenomic RNA and a minor band migrating at 3.9 kb, which corresponds with a transcript derived from the 5' viral LTR. The identity of the 11.6-kb transcript was confirmed by hybridizing the same blot with a probe derived from rat FN cDNA. This probe also detects the endogenous murine FN message (8.1 kb), which is present in rat FN expressor clones as well as in the control cell lines. The rat FN mRNA signal was ~10% of the signal for endogenous murine FN mRNA. Therefore, cDNA clones for rat FNs are readily transcribed in murine 3T3 cells under the control of the MLV-LTR promoter.

To detect and quantitate secretion of recombinant rat FNs from 3T3 cells, immunoprecipitations of supernatants harvested from [$^{35}$S]methionine-labeled cells were carried out with a mouse mAb, M9, specific for rat FN or with an anti-rat FN polyclonal serum R61. SDS-PAGE analysis of immunoprecipitates from representative clones expressing O, B, V, or BV rat FN forms. All these forms of rat FN were secreted into the medium as proteins with molecular masses around 220-250 kD. The slight differences in molecular weight are as expected from

their differences in polypeptide chain by including neither, either or both of the EIIIB and V regions. As seen most evidently in cells secreting the O form of rat FN, the endogenous mouse FN (a mixture of different forms but mostly larger than the O form) is coprecipitated with M9. This result showed that the recombinant rat FN can form dimers with the endogenous mouse FN which has been observed before for 3T3 cells expressing the COOH-terminal third of rat FNs. Schwarzbauer et al., Proc. Natl. Acad. Sci. USA 84:754-758 (1987). No endogenous FNs were precipitated by M9 from supernatants of 3T3 parental cells or clones infected with vector alone. The immunoprecipitations with the polyclonal anti-FN serum demonstrated that all these clones secreted comparable amounts of total fibronectins. Estimates from densitometry of the autoradiographs showed that the recombinant rat FNs secreted from 3T3 cell clones represent ~10% of the total FNs produced by these cells. This estimate corresponds well with the 10% rat FN transcripts compared with total fibronectin message as determined from Northern blot analysis. Thus, the chimeric rat FN mRNAs are efficiently translated and the rat fibronectins expressed in stable infected NIH 3T3 cell clones are efficiently processed, assembled into dimers and secreted into the medium. These cells also assemble the recombinant rat FNs into extracellular matrix.

Recombinant fibronectin was also expressed in WEHI231 cells. The single cell clones secreting the corresponding rat FNs were isolated by limiting dilution from the G418-resistant pools. Secretion of various forms of rat FNs was determined by immunoprecipitation

using the polyclonal antiserum R61 followed by SDS-PAGE analysis in the presence or absence of reducing agents. A major protein product migrating at 220-250 kD was immunoprecipitated from the media of [$^{35}$S] methionine-labeled cells clones expressing the O, B, A or V forms of rat FNs. The apparent molecular weights of the proteins are as expected for the various rat FN forms and also correspond with those of rat FNs expressed in NIH 3T3 cells as described above. This suggests that no major different posttranslational modifications occurred in recombinant FN synthesized in lymphoid WEHI231 cells which normally do not produce any endogenous FNs.

WEHI231 cells are lymphocytes and, thus, do not produce fibronectin. Ralph, P. Immunol. Rev., 48:107-121 (1979). Subsequent analyses demonstrated that all variants produced by WEHI231 cells bind to gelatin and heparin. For example, variants O ($B^-A^-V^-$), B ($B^+A^-V^-$), A ($B^-A^+V^-$) and V ($B^-A^-V^+$) bind both to gelatin and to heparin (as well as to a polyclonal antibody, R61.1, which recognizes total fibronectin).

All fibronectin variants produced were also shown to promote cell adhesion or spreading, in a variety of cell types. One representative experiment was performed with the mouse melanoma cell line B16F10. On control substrata coated only with bovine serum albumin (BSA), the cells either did not attach, or spready poorly. On substrata coated with a low amount of recombinant FN (in this case 2 μg/ml A form), significantly increased numbers of cells attach, but relatively few assume a well-spread morphology. When plated on higher doses of FNs (16 μg/ml A form), the majority of the cells adhered and spread well. This observation was confirmed by

quantitative measurements shown in Fig. 5 for B16F10 melanoma cells. The percentages of cells spreading are plotted as average scores for three independent experiments and the SDs were <10%. This experiment demonstrated that these four forms of recombinant FNs have similar dose-response curves in promoting B16F10 cell spreading, reaching saturating concentration at ~10 μg/ml. These results suggest that, in the basic adhesion and spreading assays with these established adherent cell lines, all forms of FN tested are equivalent.

Recombinant fibronectin variants were also shown to promote cytoskeletal organization, as assessed using antibodies against actin or vinculin. Through their transmembrane integrin receptors, extracellular FNs can induce cytoskeletal organization including organized actin bundles and focal contact formation. To examine these transmembrane effects, B16F10 melanoma cells were cultured on substrata coated with various recombinant FNs. 2 hours later, cells were fixed and stained for F actin and vinculin distribution using double-label immunofluorescence. When low concentrations of the various FNs were used, actin bundles were visualized in only a small percentage of cells while diffuse and unorganized patterns were evident for most cells. Similarly, few focal contacts were detected as determined by staining for vinculin. When plated on higher concentrations of FNs, however, extensive microfilament bundles were detected for the majority of cells and discrete focal contacts were localized at termini of actin bundles. These results indicated that recombinant FN V form was able to induce cytoskeletal organization. Similar effects were observed for the other forms of FNs obtained from WEHI231 cells (O, V, B forms).

Recombinant variants were also shown to promote reversion of Nil.8-HSV (transformed) morphology to normal cell morphology, using the method of assessing reversion described by Ali and co-workers. Ali, I. et al., Cell, 11:115-126 (1977). As shown in Figure 6a, a substantial population of NIL8.HSV cells are rounded and detached from the substrate. The cells assumed a more flattened and aligned morphology upon addition of the recombinant FN A form (Figures 6b and 6c). Similar effects were also observed for O, B, V forms of FNs. The differences in dose response among the different forms were, at most, two to threefold in different experiments. Therefore, in agreement with results obtained from basic adhesion and spreading assays using adherent cells, the ability of FN to revert the morphology of these transformed cells does not appear to reside in the EIIIB, EIIIA, or V regions.

Assessment of incorporation of recombinant fibronectin into cell matrices was also carried out and showed that all variants were incorporated and that variants $B^+$ ($B^+A^-V^-$) and $A^+$ ($B^-A^+V^-$) were incorporated somewhat more effectively than the other variants. As shown in Figure 7, recombinant rat FNs formed fibrillar networks characteristic of the usual extracellular matrix distribution of FN. Furthermore, total extracellular FN staining (with polyclonal antiserum R61) increased significantly upon addition of exogenous recombinant FNs, indicating their contribution to matrix formation. All forms of FNs incorporated into the existing matrices. However, two to threefold differences in the doses of recombinant FNs required to give a particular level of rat FN-specific fluorescence were noted. The O and V

forms required higher levels added than did the A and B forms to give equivalent staining. Minimum doses for detectable M9 staining were 10 μg/ml for B or BAV and 30 μg/ml for V. Figure 7 shows approximately equivalent incorporation of rat FN at 30 μg/ml B form (Figures 7a and 7d) and 90 μg/ml V form (Figures 7e and 7f). These results suggest that inclusion of the EIIIA or EIIIB segments characteristic of tissue FN may enhance the ability of FN to incorporate into existing matrix.

Thus, the assays carried out, using standard techniques, resulted in the determination that the recombinant fibronectin variants are produced and are biologically functional. The following is an outline of the results obtained:

Retroviral Expression of Fibronectin

| | | |
|---|---|---|
| 1. | Full length fibronectin variants are efficiently expressed. | |
| 2. | All forms are assembled and secreted. | |
| 3. | All forms bind to gelatin and heparin. | |
| 4. | Cell adhesion and spreading (BHK/B16/NIL8-HSV/CHO/RAT-1) | - ALL FORMS WORK WELL |
| 5. | Cytoskeletal organization. (BHK/B16) | - AT MOST 2-3X DIFFERENCE IN DOSE RESPONSE |
| 6. | Reversion to normal morphology. | - $A^+$ OR $B^+$ FORMS SLIGHTLY MORE EFFECTIVE |
| 7. | Migration. (NIL8-HSV) | - ALL FORMS WORK |
| 8. | Matrix Assembly. | - $A^+$ OR $B^+$ FORMS ARE MORE EFFECTIVE |

Modified Essentially Full Length Recombinant Fibronectins

Fibronectins having a portion of regions B, A, V or combinations of these can also be produced. For example, the first 25 amino acids of the V region (referred to as V25 segment) which are shown in Figure 8 can be selectively spliced out independently of the rest of the V region. The V25 segment is important in the selective adhesion of various cell types and is recognized by the integrin $\alpha_4\beta_1$ fibronectin receptor. $V^+$ recombinant fibronectins having the V25 segment alternatively spliced out can be produced as homodimers or heterodimers.

WEHI231 lymphoid cells interact with an alternatively spliced region of FN, specifically with the C-terminal 10 amino acids (GPEILDVPST) of the V25 segment. Only those forms of FN that contain this segment promote spreading of these cells, and this spreading can be blocked either by synthetic peptides from the V25 segment or by antibodies to the integrin $\alpha_4$ subunit. Furthermore, integrin $\alpha_4\beta_1$ binds specifically to the V25 peptide coupled to Sepharose.

Thus, integrin $\alpha_4\beta_1$ is an FN receptor distinct from the $\alpha_5\beta_1$ integrin receptor, which recognizes the RGDS site in FN (Pytela et al., Cell 40:191-198 (1985); Pytela et al., Science 231:1559-1562 (1986); Argraves et al., J. Cell Bio. 105:1183-1190 (1987); and Wayner et al., J. Cell Bio. 107:1881-1891 (1988)) and another integrin $\alpha_3\beta_1$, which also binds to FN at an unknown site (Takada et al., J. Cell Biochem. 37:385-393 (1988); Wayner, E.A. and W.G. Carter, J. Cell Bio. 105:1873-1884 (1987); and Wayner et al., Ibid. (1988). While $\alpha_3\beta_1$ and $\alpha_5\beta_1$ are prevalent on cultured fibroblastic cells (Helmer et al.,

J. Bio. Chem. 262:3300-3309 (1987); Wayner, E.A. and W.G. Carter, Ibid. (1987); and Wayner et al., Ibid. (1988)), and the adhesion of such cells to FN is frequently blocked by RGDS-containing peptides (Pierschbacher, M.D. and E. Ruoslahti, Nature 309:30-33 (1984); and Pierschbacher, M.D. and E. Ruoslahti, Proc. Natl. Acad. Sci. USA 81:5985-5988 (1987)), $\alpha_4\beta_1$ is expressed predominantly on lymphoid and myeloid cells (Helmer et al., J. Bio. Chem. 262:3300-3309 (1987); Helmer et al., Ibid. (1987); Wayner et al., Ibid. (1989)).

The V25 segment can be selectively spliced out independently of the rest of the V region in mammals (Schwarzbauer et al., Cell 35:421-431 (1983); Kornblihtt et al., Nucl. Acids Res. 12:5853-5868 (1985); Sekiguchi et al., Biochemistry 25:4936-4941 (1986)) and a 44 amino acid segment (V44) that includes V25 can be similarly spliced out in chickens (Norton, P.A. and R.O. Hynes, Mol. Cell. Biol. 7:4297-4307 (1987); french-Constant, C. and R.O. Hynes, Development 106:375-388 (1989)). The sequences of these segments are well conserved; the V25 segment is identical in humans, rats, and cows (Peterson et al., "Primary structure of fibronectin" In Fibronectin, D.F. Mosher, ed. (San Diego, Academis Press) (1989); and Hynes, R.O., Fibronectins (New York:Springer-Verlag) (1989)), and the segment corresponding to the V10 peptide in chickens contains 60% identical and 80% homologous residues (Norton, P.A. and R.O. Hynes, Ibid. (1987)). This conservation is consistent with an important role for this segment, and Humphries et al., J. Bio. Chem. 262:6886-6892 (1987) have previously reported that peptides corresponding to this segment will inhibit

adhesion of murine B16F10 melanoma cells when soluble and promote their adhesion when bound to a surface. They showed also that V25 peptides (CS-1) coupled to proteins will promote neurite outgrowth of chicken peripheral neurons (Humphries et al., J. Cell Bio. 106:1289-1297 (1988)) and attachment and migration of neural crest cells (Dufour et al., EMBO J., 7:2661-2671 (1988)).

However, most of the experiments on mammals do not test for selective exclusion of the V25 segment, which can be omitted independently of other parts of the V region in mammals. Given the difficulty of assaying for the absence of this segment, it is not yet possible to say whether or not V25-negative forms of FN may be found in specific locations. The absence of the V segment from 50% of plasma FN subunits is of potential relevance given the fact that many circulating blood cells express $\alpha_4\beta_1$ (Helmer et al., J. Bio. Chem. 262:3300-3309 (1987); Helmer et al., J. Bio. Chem. 262:11478-11485 (1987); Wayner et al., J. Cell Biol. 109:1321-1300 (1989)). Since plasma FN appears to be a heterodimer of $V^+$ and $V^-$ subunits (Schwarzbauer et al., J. Cell Biol., 109:3445-3453 (1989) each molecule contains only a single binding site for $\alpha_4\beta_1$. This appears to be insufficient for high affinity binding to cell surfaces since FN is not found as a surface component of circulating blood cells. If, as appears to be the case, matrix FN is largely $V^+$, it should have a higher avidity for binding of cells bearing $\alpha_4\beta_1$ integrin. This could play a role in the adhesion of various blood cells to exposed extracellular matrix, such as endothelial basement membrane. In this context, it is interesting to note that $\alpha_4\beta_1$ has recently been reported

to be involved in homing of lymphocytes to Peyer's patch high endothelial venules (Holzmann et al., Cell 56:37-46 (1989)).

In the integrin $\beta_1$ subfamily there are three FN receptors: $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_3\beta_1$ specific, respectively, for the RGDS site, 10 amino acids in the V25 segment, and an unknown site. Other integrins are also known to interact with FN, including $\alpha_{IIb}\beta_3$ (GPIIb/IIa), which binds to the RGDS site (Gardner and Hynes, Cell 42: 439-448 (1985); Pytela et al., Science 231:1559-1562 (1986); D'Souza et al., J. Bio. Chem. 263:3942-3951 (1987)) and $\alpha_v\beta_x$, which probably also binds to the RGDS site (Cheresh et al., Cell 57:59-69 (1989)).

Production of Recombinant Cellular Fibronectin

Fibronectin of the present invention can be produced, as described previously, using an appropriate vector containing cDNA encoding full-length recombinant cellular fibronectin, which is introduced into and expressed by an appropriate host cell. The DNA encoding the cellular fibronectin can be cDNA or DNA, synthesized by known methods, which has the same nucleotide sequence as the cDNA or a functional equivalent thereof (i.e., one which encodes a product having the same characteristics and exhibiting the same functions as the recombinant cellular fibronectin described herein). Appropriate host cells include, but are not limited to, NIH 3T3 and WEHI231 cells; other cells in which the complex cellular fibronectin can be produced and properly processed can also be used.

Uses of Recombinant Fibronectin Produced by the Present Method

Fibronectin produced by the process of the present invention has several therapeutic and clinical uses, and can be used wherever fibronectin is naturally utilized in the body. For example, fibronectin plays an important role in the cell migration associated with wound healing and tissue repair in general. Immunolocalization studies have shown that abundant fibronectin is present in healing wounds. In situ hybridization studies have shown that cellular fibronectin synthesized locally in wound tissue contains both the B and the A segments or regions.

Fibronectin can also be used to promote nerve regeneration in some cases. For example, while not strictly cell migration, the outgrowth of neurites from neurons involves many of the same principles and similar mechanisms. There is considerable evidence that some neurons will respond to fibronectin by extending neurites. It has been shown that fibronectin promotes outgrowth of neurites from aggregates of 7-day chick neural retinal cells. Akers, R.M. et al., Dev. Biol., 86:179-188 (1981). Fibronectin has been shown to promote neurite growth in chick ganglion cells (Carbonetto, S.T. et al., J. Neurosci., 3:2324-2335 (1983) and in human ganglion cells. (Baron-Van Evercooren, A. et al., J. Neurosci. Res., 8:179-183 (1982)).

Fibronectin also plays a role in hemostasis and thrombosis and can be used in the treatment of blood or clotting disorders. Thrombotic diseases are major killers, and the ability to control thrombosis would be a valuable therapeutic tool in treating them. Monoclonal

antibodies raised against fibronectin, or specific peptides, could be used to intervene in the interactions between the ligand and its receptors. For example, fibronectin interacts with fibrin, becomes incorporated into clots, and is crosslinked to the fibrin by factor XIIIa transglutaminase. Therefore, as the clot is formed from platelets and fibrin, fibronectin becomes an integral part of it. Intervening in this reaction by preventing fibronectin from performing its usual role provides a method of preventing dangerous blood clots. It has been shown that soon after wounding, fibronectin and fibrin appear in the area of a wound. These proteins then serve as a substrate for adhesion and migration of the cells repairing the defect and, in most cases, subsequently disappear. Fibronectin also plays a role in removal of debris by various cell types.

When a wound is made in the skin, several wound healing processes ensue. The epidermal cells migrate in to cover the wound. Concurrently, beneath the healing epidermal layer, granulation tissue forms and eventually neovascularization follows. These three processes involve different cell types and are best considered separately.

The first detectable event is the formation of a fibrin-fibronectin clot in the area of the wound. Grinnell, F. et al., J. Invest. Dermatol., 76:181-189 (1981); Clark, R.A. et al., J. Invest. Dermatol., 79:264-269 (1982); Repesh, L.A. et al., J. Histochem. Cytochem., 30:351-358 (1982). There is a large increase in fibronectin soon after wounding and the epidermal cells migrate beneath the main clot on the fibronectin-

fibrin matrix. After wound epithelialization is complete, the thickened basement membrane becomes thinner again and the fibrin and fibronectin fall to low levels and are replaced by laminin and type IV collagen typical of the normal BM. In an experiment involving wounding of rat skin implanted in mice, Clark and co-workers were able to distinguish locally produced cellular fibronectin (rat) from deposited plasma fibronectin (mouse). Clark, R.A. et al., J. Invest. Dermatol., 80:26s-30s (1983). They found that the early, provisional matrix consisted largely of plasma fibronectin which was gradually replaced by cellular fibronectin during epidermal wound healing.

Donaldson and Mahan have shown directly that fibronectin and fibrin promote epidermal migration, using implants placed in wounds in new skin. Donaldson, D.J. and J.T. Mahan, J. Cell Sci., 62:117-127 (1983) and Donaldson, D.J. and J.T. Mahan, Cell Tissue Res., 235:221-224 (1984). Coating of the implants with fibronectin or fibrin promoted migration in a dose-dependent fashion and this was specifically inhibited by antibodies to these two proteins.

Fibronectin can be used to treat corneal lesions. The cornea of the eye is a relatively simple, nonvascularized system. The cornea consists of a layer of epithelial cells on a basement membrane, beneath which lies a thick stromal layer composed largely of collagen with a few keratocytes. Beneath the stroma is a specialized basement membrane (Descemet's membrane) with a layer of endothelial cells attached to it. Fibronectin is believed to be involved in the migration of the

endothelial cells and stromal cells during development, but in the mature cornea the only significant concentration of fibronectin is in Descemet's membrane; the basement membrane of the corneal epithelium has little fibronectin. Kurkinen, M. et al., Dev. Biol., 69:589-600 (1979) and Cintron, C. et al., Curr. Eye Res., 3:489-499 (1984).

Within a few hours after a scrape wound which removes the epithelial layer, fibronectin and fibrin appear on the surface of the cornea. Between one and two days after wounding, the corneal epithelium grows back over this fibronectin and fibrin-coated surface and the fibronectin and fibrin disappear over the next several days. Fibronectin also appears in significant amounts within the stroma which normally contains only small amounts.

This pattern of appearance of fibronectin and fibrin indicates that these proteins form the substrate promoting migration of the healing epithelium. Nishida and co-workers have cultured blocks of cornea in vitro and shown that the epithelial layer migrates over the cut stromal surface. Nishida, T. et al., Jpn. J. Ophthalmol., 26:410-415 (1982) and Nishida, T. et al., Jpn. J. Ophthalmol., 26:416-424 (1982). The migrating epithelium is underlain by a layer of fibronectin. Addition of autologous serum or, more significantly, purified plasma fibronectin to the cultures accelerated the epithelial migration and anti-fibronectin antisera inhibited it. Thus, fibronectin can be used in the treatment of corneal epithelial wounds.

Fibronectin has therapeutic value in promoting corneal epithelial wound healing and in leading to curing of persistent corneal ulcers. Fibronectin can be used to prepare eye drops, for example. Treatment with these eye drops leads to accelerated healing of corneal ulcers and other defects. It has been shown that the protease inhibitor, aprotinin, has dramatic therapeutic effects on the healing of corneal lesions. Thus, combinations of fibronectin and protease inhibitors may prove particularly efficacious. Past efforts in improving wound healing have made use of plasma fibronectin, which, as mentioned, is a mixture of types. Homogeneous cellular fibronectin might be more effective in promoting wound healing (e.g., because of the presence of B and/or A regions).

Fibronectin treatment may be useful in treatment of periodontal disease, which is characterized by failure of attachment of gingival tissue to the tooth roots. Studies in vitro have shown that attachment and migration of gingival fibroblasts and periodontal ligamentum cells on teeth and bone fragments is promoted by citric acid demineralization to expose collagen and by fibronectin treatment. Terranova, V.P. and G.R. Martin, J. Periodont. Res., 17:530-533 (1982); Fernyhough, W. and R.C. Page, J. Periodontol., 54:133-140 (1983); Arisawa, Y. and Y. Abiko, Gen. Pharmacol., 15:293-239 (1984); Terranova, V.P. et al., J. Periodontol., 58:247-257 (1987); Terranova, V.P. et al., J. Periodont. Res., 22:248-251 (1987).. The fibroblasts attach and synthesize an extensive extracellular matrix attached to the tooth fragments. These results suggest that fibronectin

promotes attachment of gingival tissue to tooth roots in vivo and this appears to be the case. When tooth roots are surgically exposed and planed, connective tissue attachment during healing is significantly promoted by treatments with citric acid and fibronectin. Thus, in wound healing the fibronectin, or even conjugated peptides can be used to promote cell adhesion and migration on the wound bed. In the case of fibrosis, the accumulation of fibronectin and other matrix molecules, and the cells that produce them must be controlled, for example, by intervention in the stimulatory events between cells or in the biosynthesis of fibronectin. It is also possible to interfere with fibronectin function by the use of antibodies or peptides.

The quantity of the present fibronectin to be administered will be determined on an individual basis, and will be based at least in part on consideration of the severity of the symptoms to be treated and the result sought.

The agent or drug containing fibronectin can be administered by subcutaneous or other form of injection, intravenously, parenterally, transdermally or topically. The form in which it will be administered will depend upon the route by which it is administered.

A fibronectin composition of the present invention can optionally include other components. The components included in a particular composition are determined primarily by the manner in which the composition is to be administered. For example, a composition to be applied topically can include, in addition to fibronectin, or a derivative thereof, a binder (e.g., carboxymethyl

cellulose, gelatin), a protease inhibitor, (e.g., aprotinin), a filler (e.g., lactase) or an emulsifier. A composition to be administered dropwise (e.g., as eyedrops) may contain a liquid carrier (e.g., saline).

In general, a composition of the present invention to be applied to a skin wound, for example, would be applied directly to the wound for a period of time necessary to induce healing. More than one application may be necessary. The dosage, or concentration of fibronectin, in the composition will also vary on an individual basis and be determined by the type and severity of the symptoms to be treated.

The invention will be further illustrated by the following non-limiting Exemplification.

## EXEMPLIFICATION

### Cell Cultures

NIH 3T3 and Psi 2 cells were grown in Dulbecco's modified Eagle's medium (DME) plus 10% calf serum (CS, Gibco Laboratories, Grand Island, NY). Mouse B lymphocyte WEHI231 cells (Ralph, P. (1979), Immunol. Rev. 48:107-121) were kindly provided by D. Schatz (Whitehead Institute, MIT) and grown in RPMI 1640 medium plus 10% fetal calf serum (FCS, Gibco Laboratories). NRK, Rat1 and Ni18.HSV cells were cultured in DME with 5% FCS. BHK cells were kindly provided by F. Grinnell (University of Texas, Dallas) and maintained, in DME plus 10% FCS. Murine melanoma B16F10 cells were generous gifts of I.J. Fidler (M.D. Anderson Hospital, Houston) and cultured as described (Fidler, I.J., (1974), Cancer Res. 34:1074-1078).

Plasmid Construction

Retroviral vectors containing the 3' third of rat FN cDNA including or excluding EIIIA or V have been described previously (Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). Genomic clones were isolated from a rat genomic library in EMBL3B (Tamkun et al., (1984) Proc. Natl. Acad. Sci. USA 81:5140-5144) using segments from the 5' end of λrFN2 and subsequently the 5' ends of successive clones. The segments were subcloned in pGEM vectors (Promega Biotec), checked for repetitive sequences and used to screen lambda plaques by standard methods (Maniatis et al., (1982) Molecular Cloning Manual, Cold Spring Harbor Laboratory, Gold Spring Harbor, NY). Clones were analyzed by restriction enzyme mapping and Southern blotting and suitable fragments were subcloned into pGEM vectors for further analysis.

Genomic fragments were subcloned into a murine retroviral vector, pLJ, which is a derivative of DOL (Korman et al., (1987) Proc. Natl. Acad. Sci. USA 84:2150-2154) from which the 5' splice site has been deleted. As described elsewhere (Schwarzbauer et al., 1987) genomic fragments subcloned into such retroviral vectors are accurately spliced during generation of recombinant retrovirus. Cells derived by infection with these viruses therefore contain cDNAs derived from the genomic clones and these cDNAs can be recovered by fusion rescue. cDNA clones covering the 5' 2 kb of rat fibronectin encoding nine type I repeats and two type II repeats were prepared in this way.

Clones λrFN2 to λrFN5 cover the central part of the gene which includes all the type-III repeats (Figure 3).

cDNA clones isolated from a rat liver λgt11 library cover type-III repeats 9-15 (Schwarzbauer et al., (1983) Cell 35:421-431). cDNA clones covering type-III repeats 1-9 and the two alternatively spliced EIII repeats were obtained by passage of genomic clones through retroviral vectors.

Overlapping cDNA clones covering the 5' regions of the gene were generated from the respective genomic clones, λrFN-3, λrFN-5, λrFN-8, and λrFN-9 (Patel, R.S. et al., (1987), EMBO J. 6:2565-2572; Schwarzbauer, J.E. et al., (1987), EMBO J. 6:2673-2580), using a fusion rescue method as outlined before (Schwarzbauer, J.E. et al., (1987), EMBO J. 6:2673-2580; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). The expression vector, pDOP, was constructed from pMSV-gpt (Mann, R. et al., (1983) Cell 33:153-159) by replacement of the sequences from the Kpn I site at the 3' end of the 5' long terminal repeat to the Xho I site (Mann, R. et al., (1983) Cell 33:153-159) with those from a murine leukemia virus-based vector (DO1; (Korman, A.J. et al., (1987) Proc. Natl. Acad. Sci., USA). pDOP contains a unique BamHI cloning site followed by a fragment of pBR322, the simian virus 40 (SV40) origin and early promoter, and the $neo^r$ gene (Cepko, C.L. et al., (1984) Cell 37:1053-1062; Korman, A.J. et al., (1987) Proc. Natl. Acad. Sci., USA). The $neo^r$ gene product confers resistance to the antibiotic, G418, in mammalian cells (Davies, J. and A. Jimenez (1980), Am. J. Trop. Med. Hyg. 29:Suppl. 5, 1089-1092). The polyoma virus early region increases the plasmid copy number after transfection into Ψ2' cells.

FN cDNAs were isolated from a rat liver λgt11 library (Schwarzbauer, J.E. et al., (1983) Cell 35:421-431). BamHI and Bcl I linkers were added to the 5' and 3' ends, respectively, of an EcoRI partial-Sac II fragment of λrlf 3 containing the 3'-terminal 2400 bases of coding sequence including the 360-base variable segment (V120) plus 169 bases of the 3'-untranslated region. A 110-base-pair BamHI-Bgl II fragment from pPTHm127 (Hellerman, J.G. et al., (1984) Proc. Natl. Acad. Sci., USA 81:5340-5344) containing the 5'-untranslated and "prepro" coding sequences of parathyroid hormone was then ligated to the 5' end of the linkered FN cDNA. The hybrid cDNA was inserted into the BamHI site of pDOP. The sequence across the prepro-FN junction was confirmed (Maxam, A. and W. Gilbert (1977), Proc. Natl. Acad. Sci., USA 74:560-569). cDNAs containing the 285-base difference sequence (V95) or no additional sequences (V0) were constructed by replacing the 360-base sequence with the corresponding fragments from λrlf 4 and λrlf 6, respectively (Schwarzbauer, J.E. et al. (1983) Cell 35:421-431).

FN cDNA containing the EIII segment was obtained by passage through Ψ2 cells of a retroviral vector, DOL (Korman, A.J. et al. (1987) Proc. Natl. Acad. Sci., USA), carrying an 8-kilobase (kb) EcoRI-BamHI fragment of the FN genomic clone λrFN 2 (Tamkun, J.W. et al. (1984) Proc. Natl. Acad. Sci., USA 81:5140-5144). After infection of 3T3 cells with recombinant virus, the G418-resistant ($G418^r$) cells were fused with COS cells to induce replication from the SV40 origin, and the provirus was rescued (Cepko, C.L. et al. (1984) Cell 33:153-159). Recovered cDNA copies of this region of the FN gene were

sequenced (Maxam, A. and W. Gilbert (1977) Proc. Natl. Acad. Sci. USA 74:560-569), and all splice junctions were correct.

These cDNA clones, including or excluding EIIIB, were recombined, using unique restriction sites, with the existing 3' cDNAs to give full rise to full length cDNAs encoding rat FN. All possible combinations of EIIIB, EIIIA and V were made and the constructs confirmed by restriction mapping. These full-length cDNAs extend from a Bal I site (TGGCCA) 50 nucleotides upstream of the initiator codon of rat FN (Patel, R.S. et al., (1987), EMBO J. 6:2565-2572) to a Sac II site (CCGCGG) in the 3' untranslated region (Schwarzbauer, J.E. et al., (1983), Cell 35:421-431; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758; Patel, R.S. et al., (1987), EMBO J. 6:2565-2572). They include the entire coding region of the various forms of rat FN and the 3' untranslated region included in the earlier retroviral constructs (Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). Thus, these constructs include the natural signal and propeptide segments of rat FN to allow normal secretion and processing. The full length clones were inserted into the retroviral expression vector pLJ to generate pLJ-FN plasmids. pLJ is a derivative of pDOL (Korman, A.J. et al., (1987), Proc. Natl. Acad. Sci. USA 84:2150-2154; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758), from which the 5' splice site has been completely removed, and was generated and provided by J. Schwarzbauer. This was accomplished by restriction enzyme digestion of AGCTGGCCA (plural) with Alu I and Bal I and religation which deletes four bases (CTGG) to produce sequence AGCCA. The

deletion was confirmed by sequencing. cDNAs to be expressed are inserted at a cloning site downstream of the 5' MLV LTR. After the cloning site is an SV-40 origin/promoter/enhancer segment and the neomycin resistance ($neo^r$) gene driven by the SV-40 early promoter and a pBR322 origin of replication.

Transfection of Psi 2 Cells and Infection of NIH 3T3 and WEHI231 Cells

Establishment of virus-producing Psi2 cells and infection of NIH 3T3 and WEHI231 cells were performed. Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758; Landau, N.R. et al., (1987), Mol. Cell. Biol. 7:3237-3243. 3T3 and Ψ2 cells were grown in medium plus 10% (vol/vol) calf serum. COS cells (Gluzman, Y. (1981) Cell 23:174-182) were maintained in 10% (vol/vol) fetal calf serum. DNA transfections were performed using calcium phosphate precipitation (Graham, R. and A. Van der Eb (1973) Virology 52:456-457; Parker, B.A. and G.R. Stark (1979) J. Virol. 31:360-369). Twenty hours after glycerol shock, Ψ2 medium containing transiently produced recombinant virus was removed and filtered, and 1 ml of this virus stock was used to infect 3T3 cells in the presence of polybrene at 8 μg/ml. Several days later cells were cultured in medium containing G418 at 0.5 mg/ml. The number of $G418^r$ 3T3 colonies obtained ranged from 50 to several hundred; a subset of these was isolated and expanded for further analysis. In addition, $G418^r$ Ψ2 clones were tested for virus production. Viral titers for these clones ranged from $10^3$ to $10^5$ $G418^r$ colony-forming units/ml of supernatant. The following modifications were performed. After infection, NIH 3T3

cells were selected for neo$^r$ expression in G418 (Gibco Laboratories) at a concentration of 0.5 mg/ml. A subset of G418-resistant clones was then isolated and clones were expanded for further analysis. Infected WEHI231 cells were selected with G418 at a concentration of 3 mg/ml. The selected pool of cells was then cloned by limiting dilution. Single cell clones that produced the highest amounts of recombinant FNs, as determined by immunoprecipitations, were expanded for further analysis.

Northern Blot Analysis

Total RNA was isolated from NIH 3T3 cell clones by guanidinium thiocyanate extraction followed by centrifugation through CsCl as described by Chirgwin, J.M. et al., (1979), Biochemistry 18:5194-5199). 20 µg of RNA were electrophoresed in 0.8% agarose gels containing 1.1 M formaldehyde as described (Lehrach, H. et al., (1977), Biochemistry 16:4743-4751). After electrophoresis, gels were stained with ethidium bromide to visualize the position of 28S-18S ribosomal RNA and the relative RNA content in each lane. The RNA was then transferred to Zeta-Probe blotting membranes (Bio-Rad Laboratories, Richmond, CA), which were processed using protocols recommended by the manufacturer. Duplicate RNA samples were used for hybridizations with probes corresponding to rat FN cDNA or the neo$^r$ gene. The probes were labeled with $^{32}P$ by random priming (Feinberg, A. and B. Vogelstein, (1984), Anal. Biochem. 132:6-13).

Metabolic Labeling, Immunoprecipitation, Gelatin and Heparin Binding Assays, and SDS-PAGE

Cells were labeled for 20-24 h with media containing a reduced amount of unlabeled methionine (10% of that in

normal media) and 25 μCi/ml [$^{35}$S]methionine (Tran$^{35}$S-label, ICN Radiochemicals, Irvine, CA). Conditioned media were immunoprecipitated using either a rabbit anti-rat FN serum R61 and goat anti-rabbit IgG or a mouse monoclonal anti-rat FN M9 (a generous gift of M. Chiquet) and goat anti-mouse IgG, as described (Choi, M. and R.O. Hynes, (1979), J. Biol. Chem. 254:12050-12055). Immunoprecipitates were analyzed either with or without reduction by electrophoresis through SDS-PAGE followed by fluorography. Direct binding of FNs in the conditioned media to gelatin-coupled Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was carried out as described. The bound materials were also eluted by 4 M urea in PBS and used directly for binding assays with heparin-coupled Sepharose (Pharmacia Fine Chemicals) as described by Price, J. and R.O. Hynes, (1985), J. Neurosci. 5:2205-2211.

Purification of Recombinant Rat FNs

Recombinant FNs produced from expressor WEHI231 cell clones were purified by affinity-chromatography using a gelatin-coupled Sepharose column as described by Engvall, E. and E. Ruoslahti, (1977), Int. J. Cancer 20:201-205. Briefly, the expressing clones were grown to saturation in 3 liters of growth medium. The cells were then washed with PBS and resuspended in 10 liters of RPMI 1640 plus 5% FCS that had been passed through gelatin-Sepharose 4B to deplete FN in the serum. The cells were incubated further for 3 d and the conditioned media were concentrated and subsequently loaded onto a

gelatin-Sepharose 4B column. Recombinant FNs were eluted using 4 M urea in CAPS buffer (10 mM CAPS (cyclohexylaminopropane sulfonic acid), 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, pH 11). The peak fractions as determined by UV absorption at 280 nm were pooled and dialyzed against CAPS buffer to remove urea. The final concentrations were determined again by UV absorption and also confirmed by comparison with protein standards on SDS-PAGE followed by Coomassie blue staining. Typical yields were 3-5 mg of purified recombinant FN from 10 l of culture supernatant.

Cell Spreading Assay

The biological activities of recombinant FNs were determined by a quantitative cell spreading assay modified from that described by Yamada, K.M. and D.W. Kennedy, (1984), J. Cell. Biol. 80:492-498 and Obara, M. et al. (1988), Cell 53:649-657. Restricted areas of tissue culture plates were incubated with 25 µl of serial dilutions of recombinant FNs for 2 h at room temperature, followed by incubation with 2 mg/ml heat-treated bovine serum albumin (BSA) (10 min at 80°C) in PBS for 2 hr at 37°C and extensive washing with PBS. Adherent cell lines were harvested by brief trypsinization and then washed with PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were added to coated plates at $10^5$/ml in growth media without serum. Suspension WEHI231 cells were washed with PBS and added at $2 \times 10^5$/ml. After 2 hr of incubation at 37°C, the plates were washed with PBS and fixed in 3.7%

formaldehyde in PBS. Percent cell spreading was then determined by counting three random fields (200-300 cells) using a Nikon inverted phase-contrast microscope.

Other FN Functional Assays

The effect of various recombinant FNs on Ni18.HSV cells was examined as described by Ali, I.U. et al. (1977), Cell 11:115-126. Two ml of growth medium containing 2 x $10^5$ cells were seeded in 35mm dishes. After 48 h, FNs were added in 100 µl PBS to give the desired concentration. Photographs were taken on the Nikon inverted phase-contrast microscope 24 h later.

To examine their effects on cytoskeletal organization, recombinant FNs were used to coat coverslips at various concentrations. Murine melanoma B16F10 cells or fibroblastic BHK cells were then plated onto the coated coverslips in the absence of serum. 2 h later, the distribution of actin bundles and vinculin was visualized by immunofluorescence. Briefly, cells were rinsed twice in PBS and fixed for 15 min in a freshly prepared 4% solution of paraformaldehyde (Fluka Chemical Co., Bern, Switzerland) in PBS, rinsed and permeabilized with 0.5% NP-40 in PBS for 15 min. Cells were stained with mouse mAb against vinculin (Sigma Chemical Co.) in 10% normal goat serum in PBS for 30 min at 37°C. After three washes with PBS, the second antibody mixture (rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated phalloidin in 10% normal goat serum in PBS, Cappel Laboratories, Cochranville, PA) was added and incubated for 30 min at 37°C. After three washes, coverslips were mounted in Gelvatol and examined using a Zeiss Axiophot

St. Louis, MO) of NIH 3T3 cells was as described previously (Hynes, R.O., (1973), Proc. Natl. Acad. Sci., USA 70:3170-3174). For WEHI231 cells, a modified method was employed: $2 \times 10^8$ cells were washed with $PBS^+$ (PBS plus 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and resuspended in 1 ml of $PBS^+$ containing 10 mM D-glucose (EM Science, Cherry Hill, NJ). Two millicuries of $Na^{125}I$ and a mixture of lactoperoxidase and glucose oxidase were then added to initiate the reaction (final concentrations: 20 μg/ml and 0.1 U/ml, respectively; Sigma). The labeling was allowed to continue for 10 min at room temperature with occasional rocking. Cells were then washed twice with $PBS^+$ containing 150 mM NaI and three more times with $PBS^+$.

Iodinated cells were extracted with 0.5% NP-40, and immunoprecipitation was performed as described previously (Marcantonio, E.E. and R.O. Hynes, (1988), J. Cell. Biol. 106:1765-1772). To detect integrin $\alpha_4$, extracts were immunoprecipitated using Sepharose coupled with the rat monoclonal antibody R1-2, followed by direct recovery by boiling for 3 min in the sample buffer (2% SDS, 100 mM Tris-HCl [pH 6.8], 10 mM EDTA, 10% glycerol and bromophenol blue). For some experiments, integrin complexes extracted from labeled WEHI231 cells were first dissociated by heating at 100°C for 2 min in 1% SDS. After cooling, a 5-fold excess of Triton X-100 was added, and the extracts were precipitated with antiserum against $\beta_1$ as described above. SDS-PAGE was performed by the method of Laemmli, U.K. (1970), Nature 227 680-685. Separation gels were 7% acrylamide with a 3% stacking gel.

Direct Binding Assay and Affinity Chromatography Using V25-Sepharose

The V25 peptide was covalently coupled to CNBr-activated Sepharose 4B at 2 mg peptide per ml beads according to instructions provided by the manufacturer (Pharmacia Biochemicals Co., Piscataway, NJ). For direct binding assays, iodinated WEHI231 cells were extracted using 200 mM n-octyl-$\beta$-D-glucopyranoside in 50 mM Tris[pH 7.5], 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (TBMMC) for 30 min on ice. These extracts were incubated with V25-Sepharose beads for 3 hr at 4°C, followed by washing in 50 mM n-octyl-$\beta$-D-glucopyranoside in TBMMC (washing buffer) four times. The bound material was then eluted with the sample buffer and subjected to SDS-PAGE as described above.

For affinity chromatography, 2 ml of extracts from $^{125}I$-labeled WEHI231 cells were loaded onto 1 ml (packed volume) V25-Sepharose by incubation at 4°C for 1 hr, followed by washing with 30 ml of washing buffer. Columns were eluted with 2 ml of washing buffer containing 1 mg/ml V14 peptide, followed by 1 ml of washing buffer, 2 ml of washing buffer containing 1 mg/ml V10 peptide, and 2 ml of washing buffer. Fractions of 400 $\mu$l were collected, and 40 $\mu$l of each fraction were analyzed by SDS-PAGE. Peak fractions eluted by the V14 and V10 peptides were pooled, immunoprecipitated and analyzed by SDS-PAGE, along with the starting material, as described above.

Integrin Receptor Binding

Peptide inhibition experiments were performed to define the active site in the V segment for interaction with WEHI231 cells and thus to facilitate the identification of a receptor from these cells. Its effect on WEHI231 cell spreading was examined by including the soluble peptide in spreading assays on saturating amounts of the V form of FN (60 μg/ml). The V25 peptide inhibited the spreading of WEHI231 cells in a dose-dependent manner. At a concentration of 300 μg/ml, the inhibition was greater than 80%. By contrast, the peptide had little effect on the spreading of NIH 3T3 cells on FN, demonstrating its specificity. The effect of peptides GRGDSP and GRGESP on WEHI231 cell spreading was also examined. Neither of these two peptides significantly inhibited WEHI231 cell spreading on the V form of FN, although the GRGDSP peptide blocked the attachment and spreading of NIH 3T3 cells on FN as expected.

Shorter peptides from within this segment were synthesized and tested for their ability to inhibit WEHI231 cell spreading (Figure 9). Whereas a 10 amino acid peptide (V10) comprising the C-terminal segment of the V25 peptide was almost as effective an inhibitor of spreading as the V25 peptide itself, two other 10 amino acid peptides, V10a and V10b, were ineffective in inhibition, as were two scrambled peptides, V10S1 and V10S2, which contained the same amino acids as V10 but in different orders (Figure 9). In these experiments, a 14 amino acid peptide, V14, which overlaps partially with V10, and GRGDSP both showed slight inhibition of spreading. These data localize the site within V25 that

is necessary for promoting cell spreading to a 10 amino acid segment.

It is known that integrin $\alpha_5\beta_1$ is a major functional FN receptor on many cells including NIH 3T3 cells (Solowska et al. 1989) and it recognizes the central binding domain of FN (Pierschbacher, M.D., and Ruoslahti, E., Nature, 309:30-33 (1984); Pierschbacher, M.D., and Ruoslahti, E., Proc. Natl. Acad. Sci. USA, 81:5985-5988 (1984); Pytela, R., et al., Cell, 40:191-198 (1985); Ruoslahti, E., and Pierschbacher, M.D., Science, 238: 491-497 (1987); Buck, C.A., and Horwitz, A.F., Annu. Rev. Cell Biol., 3:179-205 (1987); Hynes, R.O., Cell, 48: 549-554 (1987)). This can explain the similar degrees of spreading of NIH 3T3 cells on all forms of recombinant FNs since the central cell binding domain is present in all forms. On the other hand, it appears that a different FN receptor is expressed on the surface of WEHI231 cells, which can only interact with the V form of FN, through recognition of the alternatively spliced V25 segment.

The surface integrin expression on WEHI231 cells in comparison with that on NIH 3T3 cells were characterized. Cells were surface labeled with $^{125}I$, and detergent extracts were immunoprecipitated with several different antibodies. Rabbit antiserum for integrin $\beta_1$ was raised against a $\beta_1$ cytoplasmic domain peptide and reacts specifically with the $\beta_1$ subunit from many species, including mouse (Marcantonio, E.E., and Hynes, R.O., J. Cell Biol., 106:1765-1772 (1988)). The antiserum precipitated $\beta_1$ subunits (around 120 kd) together with the larger $\alpha$ subunits from both NIH 3T3 and WEHI231 cells.

effect on the binding. The control peptides V10a, V10b, and V10s2 (see Figure 9b) were also ineffective in blocking binding. These results parallel the effectiveness of these peptides in inhibiting WEHI231 cell spreading on the V form of FN (Figure 9). In these direct binding experiments, the scrambled control peptide, V10s1, inhibited partially.

Taken together, these results support the hypothesis that the V25 peptide is the active site for mediating WEHI231 cell spreading rather than inhibiting the cell spreading by interfering with a nearby site. The parallelism between the ability of various peptides to inhibit WEHI231 cell spreading and their ability to interfere with the binding of the 120-150 kd proteins to V25-Sepharose strongly suggests that the 120-150 kd proteins are the cell surface receptor mediating the spreading.

To test whether the 120-150 kd proteins are integrin complex $\alpha_4\beta_1$, V25-Sepharose column was then used to purify the receptor by affinity chromatography. WEHI231 cells were iodinated and extracted with n-octyl glucoside in buffer containing divalent cations. The extracts were loaded onto the V25-Sepharose column by incubating with the beads for 1 hour at 4°C and then washed extensively. The bound materials were eluted sequentially with buffers containing 1 mg/ml V14 peptide and 1 mg/ml V10 peptide. Aliquots of each fraction were analyzed on SDS-PAGE. Consistent with the direct binding experiments, proteins migrating as a 120-150 kd smear on the gel were bound to V25-Sepharose beads. About 40% of the bound material (as estimated by cpm) was eluted with the V14 peptide, and the remainder was eluted with the V10 peptide.

Furthermore, the same bound material could be eluted completely with either the V25 peptide or the V10 peptide when applied first. Fractions from both V14 peptide eluates (peak I) and V10 peptide eluates (peak II) were then pooled and analyzed along with the starting material by immunoprecipitation with several antibodies specific for integrin subunits. The antibodies against either the integrin $\beta_1$ or $\alpha_4$ subunits precipitated the 120-150 kd proteins from both peak I and peak II, as well as from the starting material. These results suggested that the 120-150 kd proteins bound to V25-Sepharose beads were indeed the integrin $\alpha_4\beta_1$ complex. The results indicate that integrin $\alpha_4\beta_1$ is a functional FN receptor and recognizes the alternatively spliced segment V25. This conclusion was confirmed for $\alpha_4\beta_1$ complexes isolated from murine melanoma B16-F10 cells. The results also suggest that the interaction between integrin $\alpha_4\beta_1$ and the V25 peptide occurs in the V10 peptide region but could be affected partially by the overlapping V14 peptide.

To prove that the interaction between integrin $\alpha_4\beta_1$ and the V25 peptide in the V form of FN is responsible for mediating WEHI231 cell spreading on the V form of FN, an antibody blocking experiment was carried out. WEHI231 cells were preincubated with the rat monoclonal antibody R1-2 specific for integrin $\alpha_4$, anti-IgM or rat IgG antibodies for 30 min at 4°C. The cells were then seeded in the dishes coated with saturating amounts (60 $\mu$g/ml) of the V form of FN. The percentage of cell spreading was then quantitated. At two different concentrations, R1-2 almost completely abolished the spreading of WEHI231 cells (>95% reduction). In contrast, spreading of

control samples incubated with Rat IgG was not affected significantly. Furthermore, incubation with a rat monoclonal antibody against mouse IgM, a major surface protein of WEHI231 cells (Ralph, P., Immunol. Rev. 48:107-121 (1979)), did not inhibit its spreading appreciably. This excluded the possibility that R1-2 blocked WEHI231 cell spreading simply by binding to their surfaces. Since R1-2 is a monospecific antibody for mouse integrin $\alpha_4$, these results prove that lymphoid WEHI231 cells respond to the V form of FN in vitro by their surface integrin receptor $\alpha_4\beta_1$.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

## CLAIMS

1. A method of producing a homogeneous recombinant cellular fibronectin homodimer that is biologically active, comprising introducing into an appropriate host cell a recombinant vector comprising a full length cDNA encoding cellular fibronectin of mammalian origin and maintaining cells containing the recombinant vector under conditions appropriate for expression of the cellular fibronectin.

2. A method of Claim 1, wherein the cellular fibronectin of mammalian origin is rat cellular fibronectin or human cellular fibronectin.

3. A method of producing a recombinant cellular fibronectin which is a homodimer, comprising transfecting an appropriate host cell with a recombinant retrovirus comprising full length cDNA encoding a cellular fibronectin of mammalian origin and maintaining cells transfected with the recombinant retrovirus under conditions appropriate for integration of the cDNA into host cell genomic DNA and expression of the integrated cDNA.

4. A method of Claim 3, wherein the host cell is NIH 3T3 cells or WEHI231 cells.

5. A method of Claim 4, wherein the cellular fibronectin of mammalian origin is rat cellular fibronectin or human cellular fibronectin.

6. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by the method of Claim 1.

7. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by the method of Claim 3.

8. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by a method comprising introducing into an appropriate host cell a recombinant vector comprising a full length cDNA encoding cellular fibronectin of mammalian origin and maintaining cells containing the recombinant vector under conditions appropriate for expression of the cellular fibronectin.

9. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, in which region EIIIB is not present.

10. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, in which region EIIIA is not present.

11. A recombinant retrovirus comprising a full length cDNA encoding cellular fibronectin of mammalian origin.

12. A recombinant retrovirus of Claim 11, which further comprises a gene encoding a selectable marker.

13. A recombinant retrovirus of Claim 13, wherein the gene encodes G418 resistance.

14. A recombinant cellular fibronectin of mammalian origin in which the carboxy terminal 25 amino acids of region V are not present.

15. A recombinant cellular fibronectin of Claim 14, wherein the fibronectin is a heterodimer or homodimer.

# Fibronectin and its variants

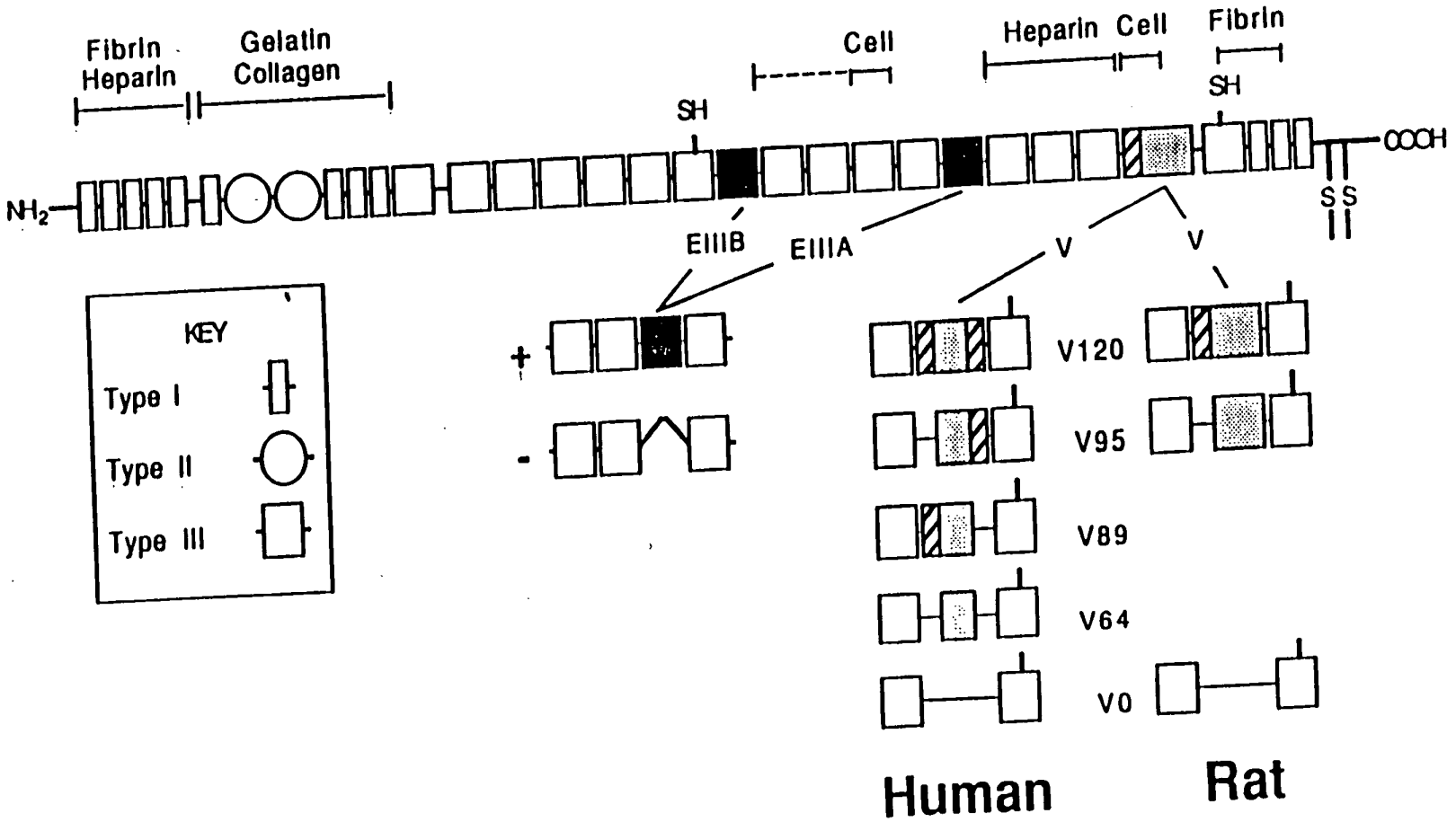



FIG. 1

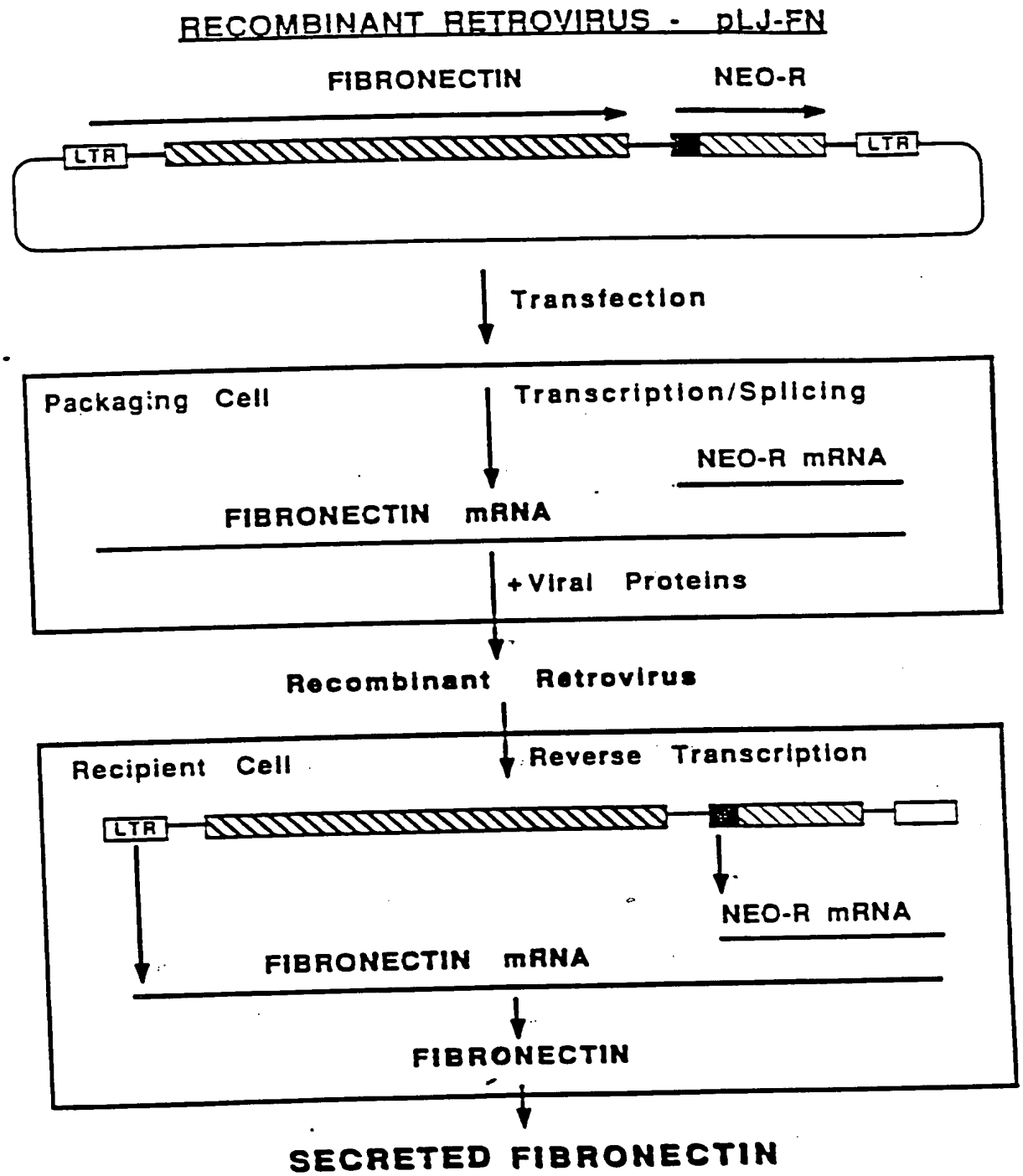
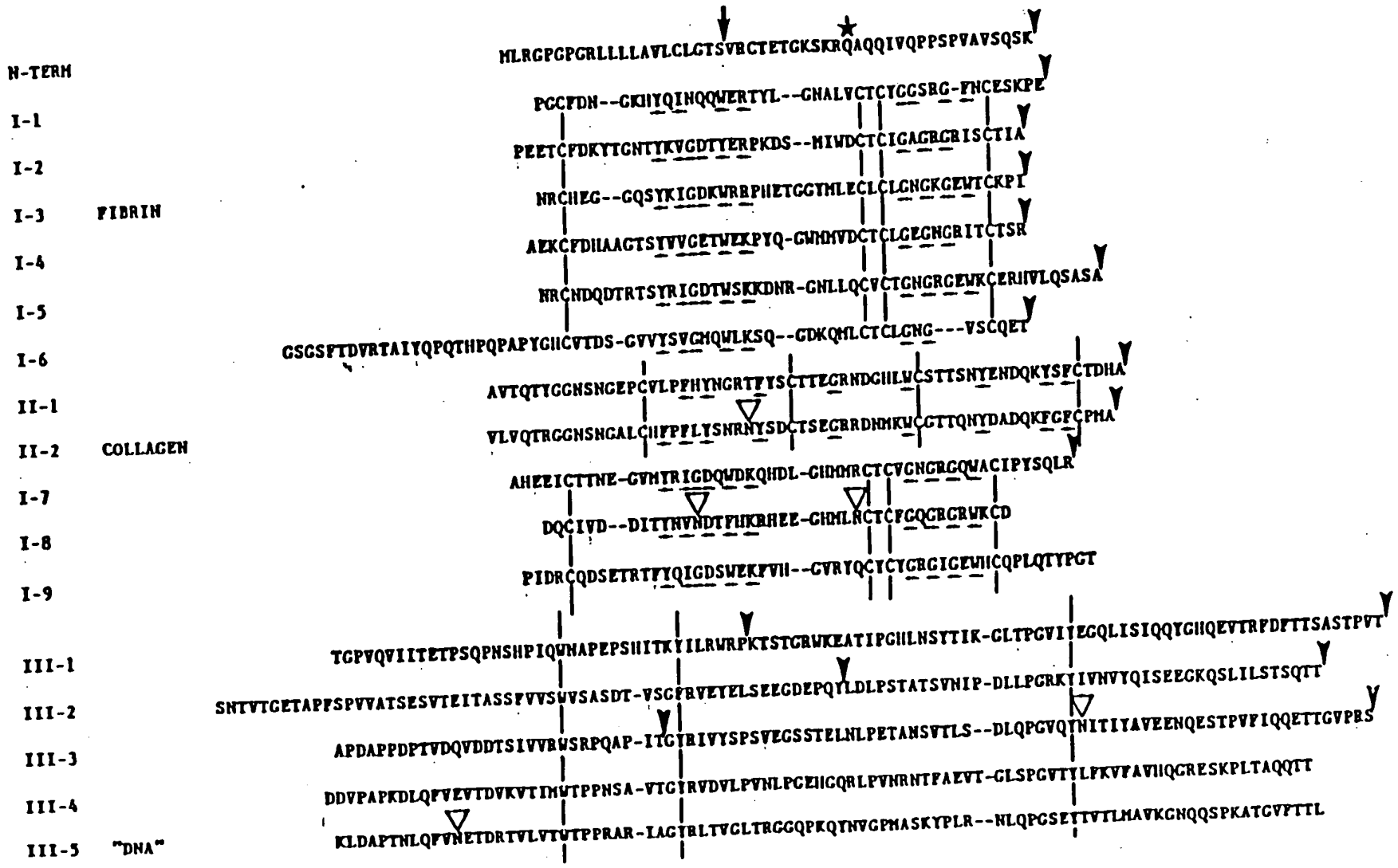
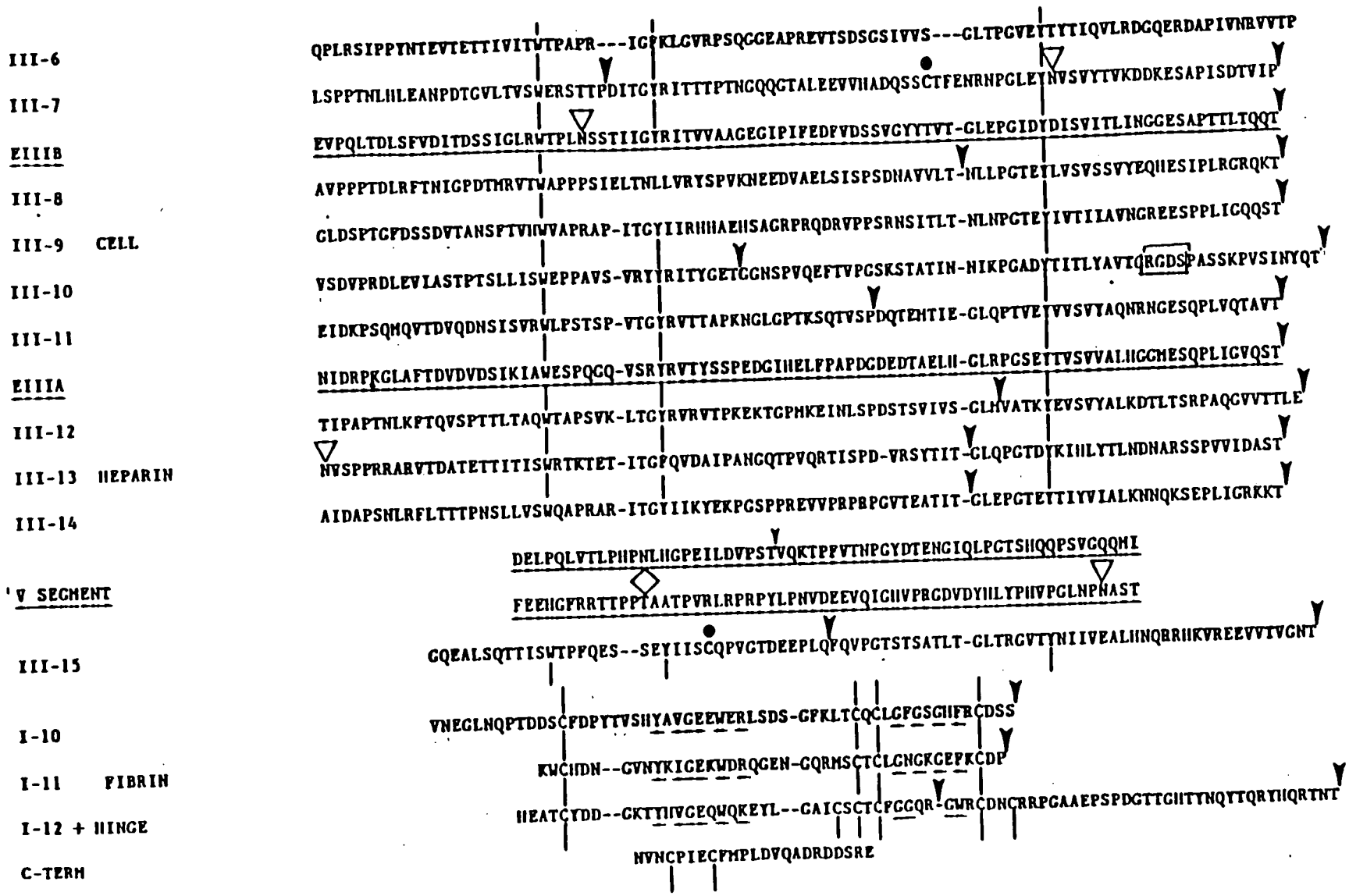

RECOMBINANT RETROVIRUS - pLJ-FN
FIBRONECTIN
NEO-R
LTR
LTR
Transfection
Packaging Cell
Transcription/Splicing
NEO-R mRNA
FIBRONECTIN mRNA
+Viral Proteins
Recombinant Retrovirus
Recipient Cell
Reverse Transcription
LTR
NEO-R mRNA
FIBRONECTIN mRNA
FIBRONECTIN
SECRETED FIBRONECTIN

FIG. 2

# FIG.3


RAT FIBRONECTIN SEQUENCE
N-TERM
I-1
I-2
I-3
FIBRIN
I-4
I-5
I-6
II-1
II-2
COLLAGEN
I-7
I-8
I-9
III-1
III-2
III-3
III-4
III-5
"DNA"

# FIG.3B CONT.'


III-6
III-7
EIIIB
III-8
III-9 CELL
III-10
III-11
EIIIA
III-12
III-13 HEPARIN
III-14
'V SEGMENT
III-15
I-10
I-11 FIBRIN
I-12 + HINGE
C-TERM

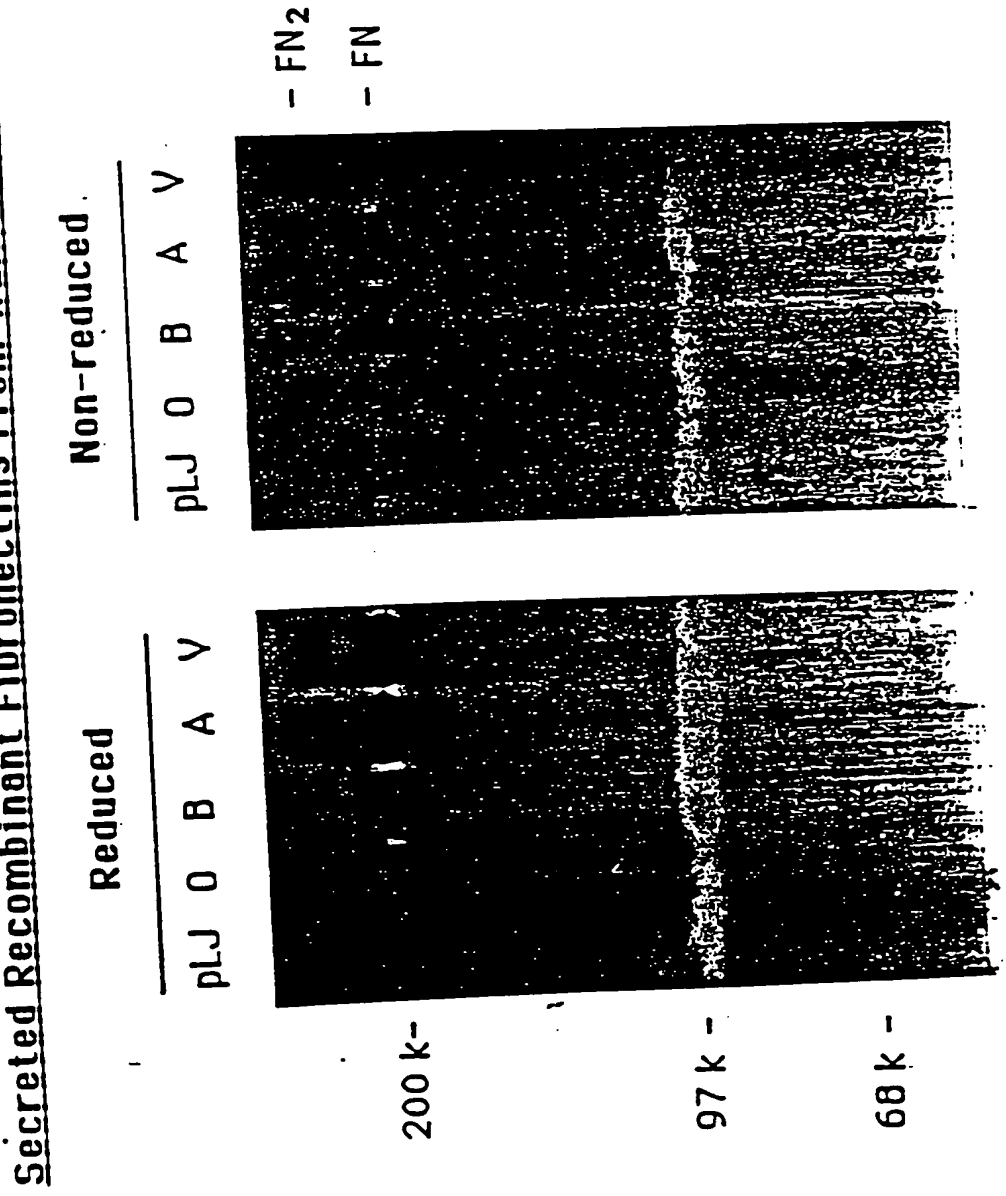

Secreted Recombinant Fibronectins From WEHI231 Cells
Reduced
pLJ O B A V
Non-reduced
pLJ O B A V
- FN2
- FN
200 k-
97 k -
68 k -

FIG.4a

FIG.4b

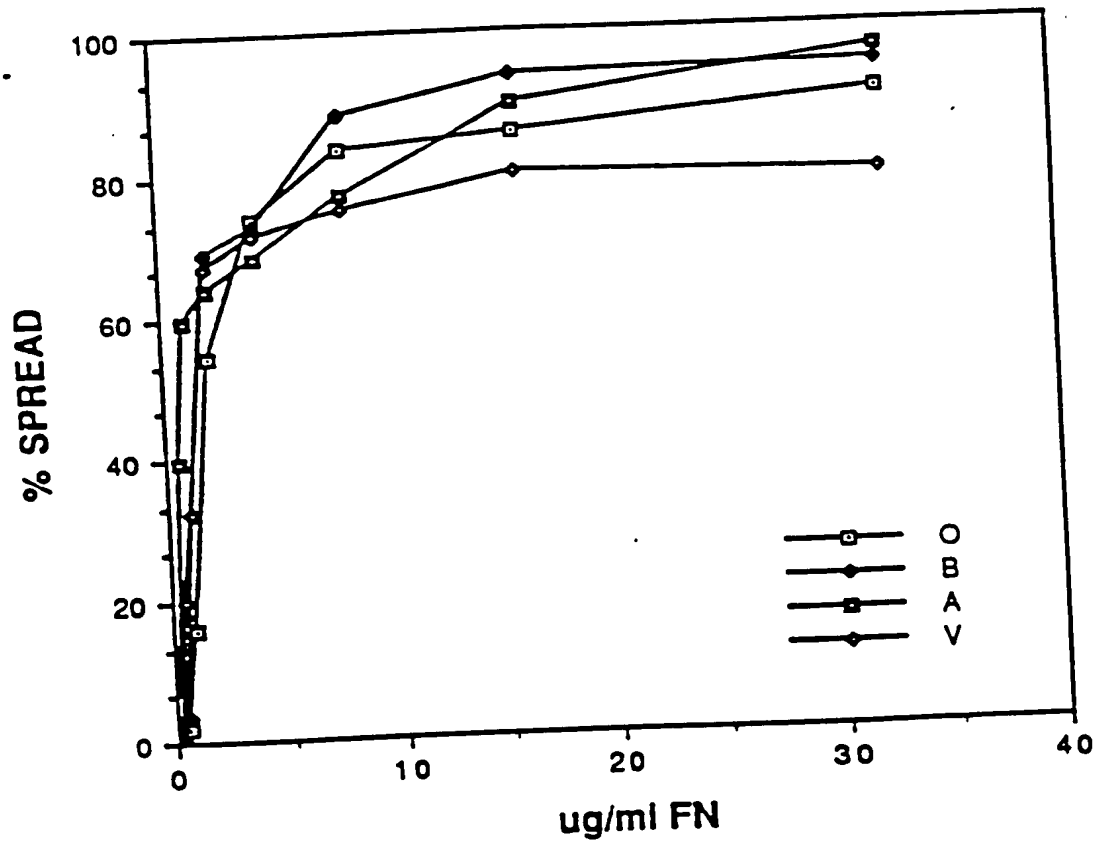



FIG. 5

Nil.8-HSV

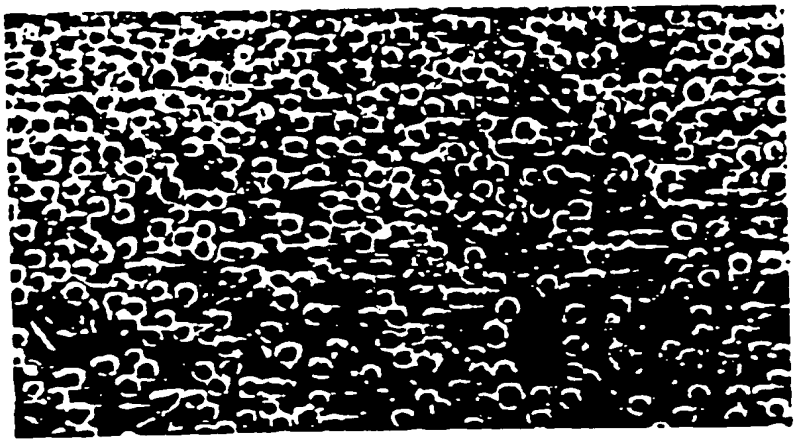


FIG.6a

—

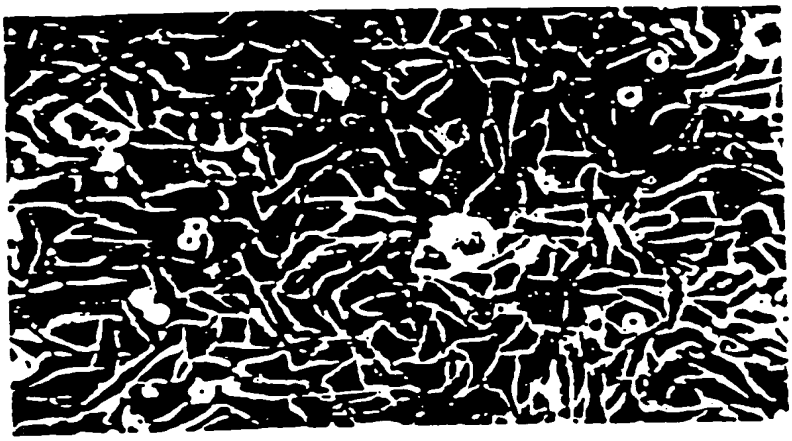


FIG.6b

10 ug/ml

A

FIG.6c

40 ug/ml

A

Total FN

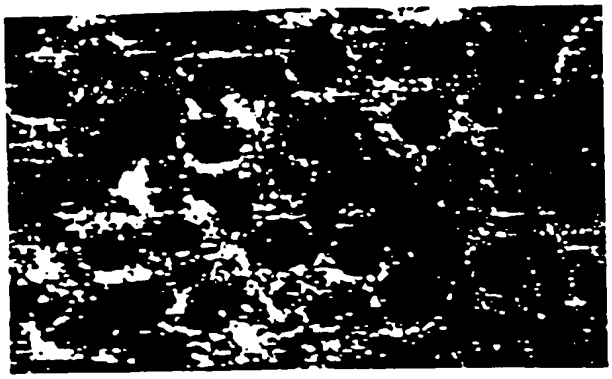


FIG.7a

Rat FN

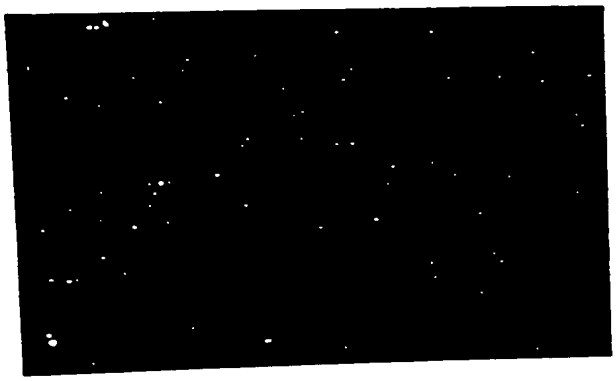


FIG.7b

Total FN

30ug/ml

B

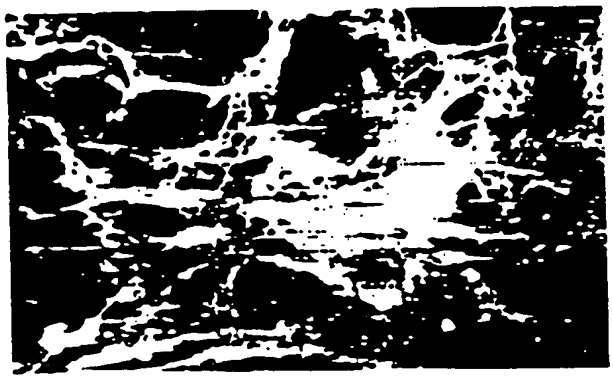


FIG.7c

Rat FN

30ug/ml

B

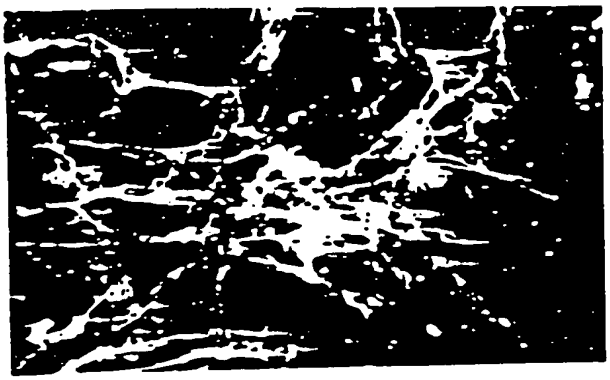


FIG.7d

Total FN

90ug/ml

V

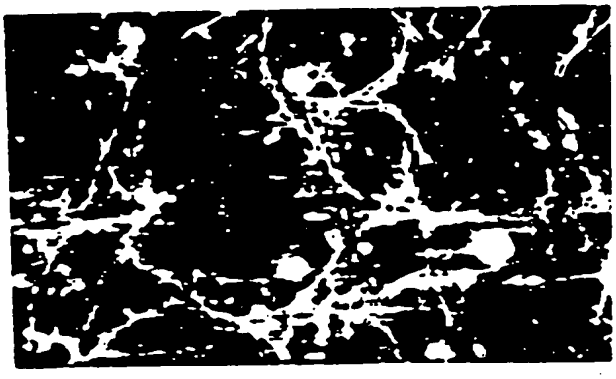


FIG.7e

Rat FN

90ug/ml

V

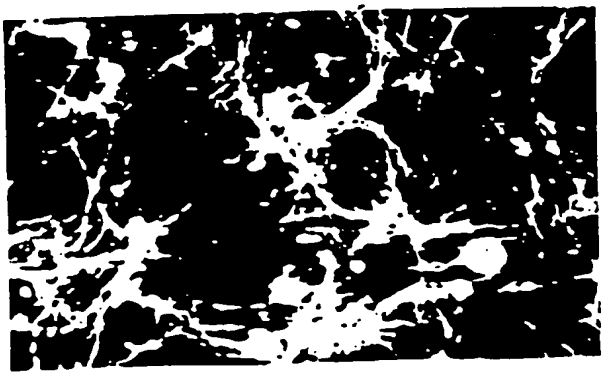


FIG.7f

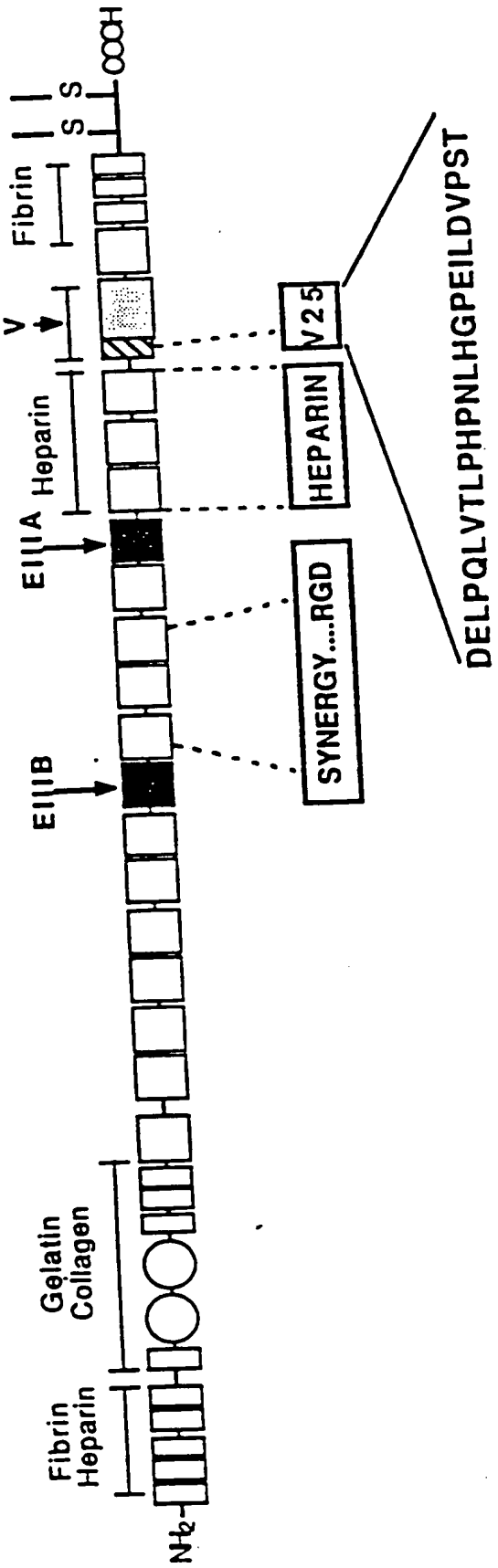

COOH
S S
Fibrin
V
EIIIA
Heparin
EIIIB
Gelatin
Collagen
Fibrin
Heparin
NH2
V25
HEPARIN
SYNERGY....RGD
DELPQLVTLPHPNLHGPEILDVPST

FIG. 8

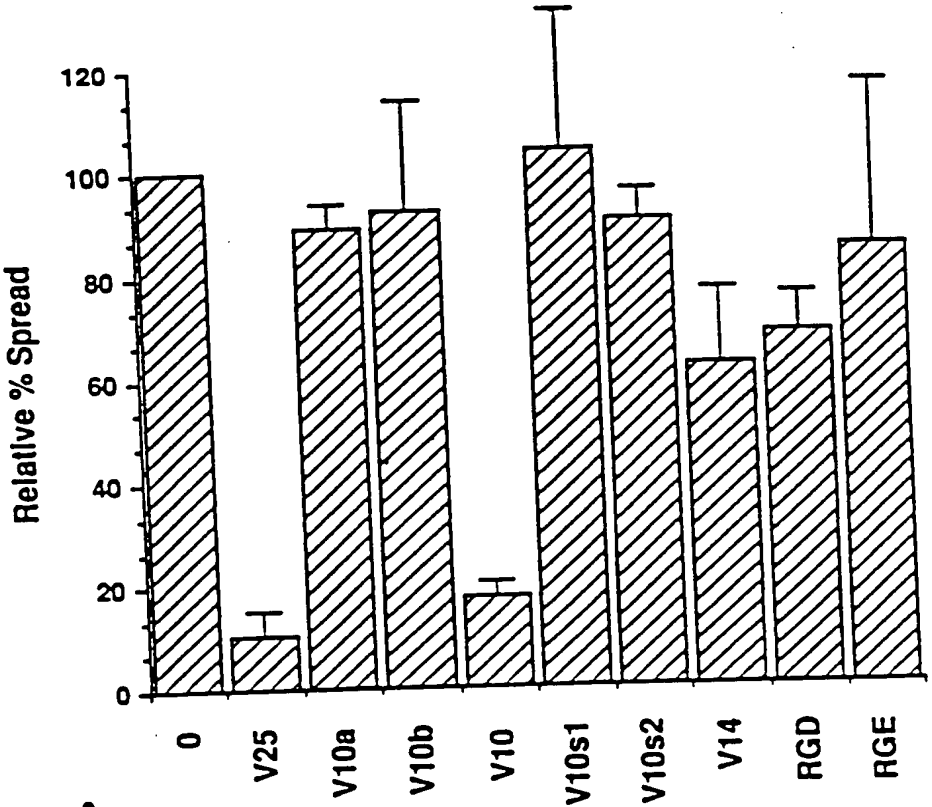

Relative % Spread
120
100
80
60
40
20
0
0
V25
V10a
V10b
V10
V10s1
V10s2
V14
RGD
RGE

FIG. 9A

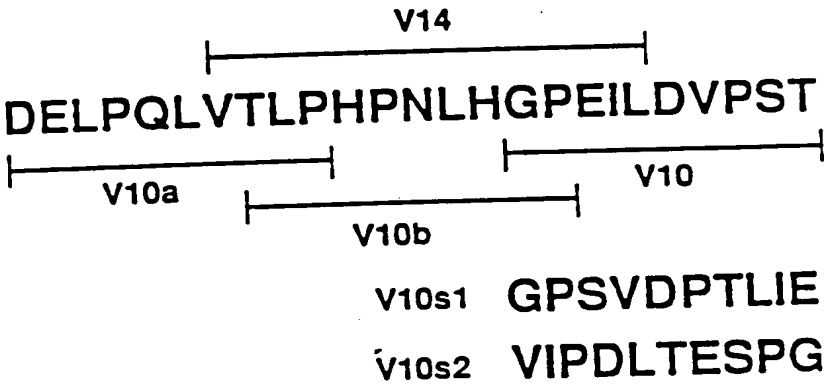

V14
DELPQLVTLPHPNLHGPEILDVPST
V10a
V10b
V10
V10s1 GPSVDPTLIE
V10s2 VIPDLTESPG

FIG. 9B

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00650

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 P 21/02, C 07 K 13/00, C 12 N 15/12, C 12 N 15/86

## II. FIELDS SEARCHED

Minimum Documentation Searched *

| Classification System | Classification Symbols |
|---|---|
| IPC[5] | C 12 N, C 12 P |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *

## III. DOCUMENTS CONSIDERED TO BE RELEVANT *

| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages ** | Relevant to Claim No. ** |
|---|---|---|
| Y | EP, A, 0207751 (DELTA BIOTECHNOLOGY) 7 January 1987 see claims | 1-15 |
| Y | EMBO Journal, volume 4, no. 7, 1985, IRL Press Limited, (Oxford, GB), A.R. Kornblihtt et al.: "Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single single gene", see pages 1755-1759 | 1-15 |
| Y | EMBO Journal, volume 6, no. 9, 1987, IRL Press Limited, (Oxford, GB), J.E. Schwarzbauer et al.: "Multiple sites of alternative splicing of the rat fibronectin gene transcript", pages 2573-2580 see page 2576, column 2, lines 3-7 ./. | 1-15 |

* Special categories of cited documents: **
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 4th May 1990 | 13. 06. 90 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | M. Peis / M. PEIS |

Form PCT/ISA/210 (second sheet) (January 1985)

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|---|---|---|
| Category * | Citation of Document, ** with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
| P,X | The Journal of Cell Biology, volume 109, no. 6, pt. 2, December 1989, The Rockefeller University Press, (New York, NY, US), J.E. Schwarzbauer et al.: "Selective secretion of alternatively spliced fibronectin variants", see pages 3445-3453 -- | 14,15 |
| A | Proc. Natl. Acad. Sci. USA, volume 84, April 1987, (Washington, D.C., US), A.J. Korman et al.: "Expression of human class II major histocompatibility complex antigens using retrovirus vectors", see pages 2150-2154 -------- | 11-13 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9000650 SA 34681

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The memoers are as contained in the European Patent Office EDP file on 12/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A- 0207751 | 07-01-87 | AU-A- 5931586 | 08-01-87 |
| | | JP-A- 62089699 | 24-04-87 |

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International Bureau

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C12N 15/12, 15/86

A1

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Not Furnished 12 January 1990 (12.01.90) US

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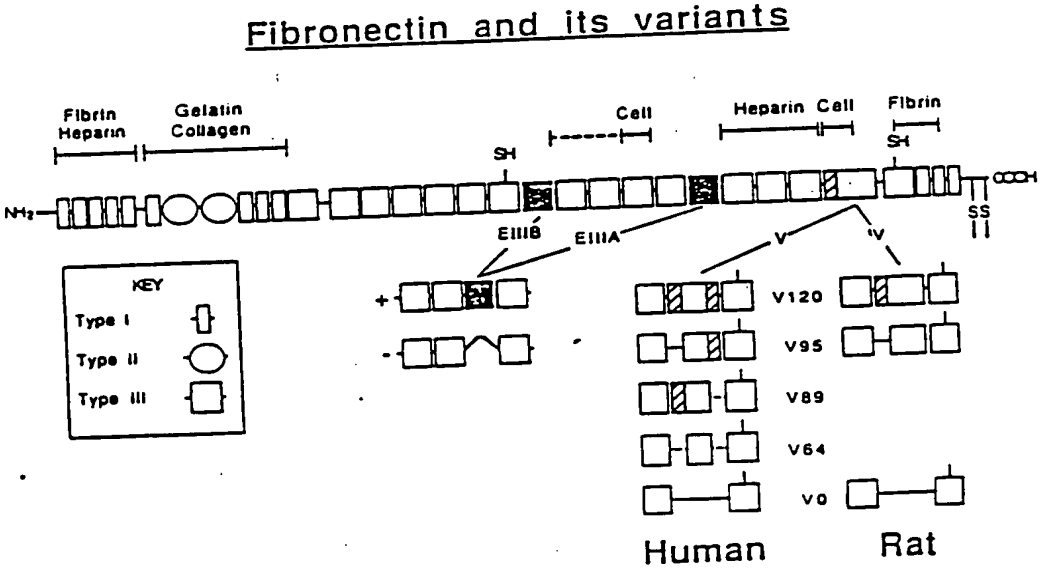


(57) Abstract

A method of producing homogeneous cellular fibronectin of mammalian origin, which is a homodimer, as well as recombinant cellular fibronectin. They can be produced having all or a portion of regions B, A, V or combinations of these. Specifically homodimers and heterodimers can be produced having the carboxy terminal 25 amino acids of region V deleted. The recombinant cellular fibronectin is useful in any application in which naturally-occurring fibronectin can be used.

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# EXPRESSION OF RECOMBINANT FIBRONECTIN IN GENETICALLY ENGINEERED CELLS

## Background

The interactions of cells with one another and with extracellular materials (e.g., matrices, solid surfaces) are of vital importance for cell function. These interactions have major effects on the proliferation, differentiation, and organization of cells. These interactions are often mediated by a class of high molecular weight glycoproteins that are involved both in these interactions and in the actual structure of extracellular matrices. One important glycoprotein of this class is fibronectin. Hynes, R.O., Ann. Rev. Cell. Biol., 1:67-90 (1985); Hynes, R.O., Scientific American, 254:42-51 (1986); Hynes, R.O. and K.M. Yamada, J. Cell. Biol., 95:369-377 (1982).

Fibronectins are high molecular weight glycoproteins involved in cell adhesion, morphology and migration. Fibronectin has been shown to consist of a dimer of two subunits, each about 250 kilodaltons in size. The two subunits, which are similar, but not necessarily identical, each fold into an elongated and flexible arm.

They are joined by disulfide bonds very near their C-termini.

Each subunit is made up of a series of tightly-folded globular domains, each of which is specialized for binding to other molecules or to cells. Hynes, R., Ann. Rev. Cell. Biol., 1:67-90 (1985). They are composed of a series of homologous repeating units of three types: Type I and II homologies, which are disulfide-bonded loops each 45-50 amino acids long, and Type III homologies, which are 90 amino acids long and lack disulfide bonds. Patel, R.S. et al., The EMBO Journal, 6:2565-2572 (1987). Hynes, Ann. Rev. Cell. Biol., 1:67-90 (1985). Recombinant DNA analyses have shown that although different subunits differ in parts of their primary sequence, they arise from a single gene and are identical over much of their sequence. Kornblihtt et al., Proceedings of the National Academy of Sciences, USA, 80:3218-3222 (1983). More than 90% of the sequence of fibronectin is made up of repeats of these three homologies. However, studies have indicated that fibronectins from different sources, e.g., fibroblasts (cellular) and plasma, are not identical. For example, plasma fibronectin contains subunits of two different mobilities on SDS - polyacrylamide gels and fibronectin from fibroblasts (cellular fibronectin) shows a different subunit pattern. Paul, J.I. et al., J. Biol. Chem., 261:12258-12265 (1986).

Recently, it has been confirmed that two Type III repeats, designated EIIIA and EIIIB, are alternatively spliced and are each encoded by a single exon. Both EIIIA and EIIIB are always omitted by liver cells,

although both can be included by other cell types and all possible combinations occur. Because both EIIIA and EIIIB are omitted by hepatocytes, neither repeat occurs in plasma fibronectin. It is interesting to note that a third region, designated V, is also alternatively spliced. Unlike EIIIA and EIIIB, however, it is alternatively spliced both in fibroblasts and hepatocytes. Schwarzbauer, J.E. et al., The EMBO Journal, 6:2573-2580 (1987). Thus, the V region can be present in both plasma fibronectin and fibroblast or cellular fibronectin. For cellular fibronectin, therefore, there are eight possible combinations or variants of these three alternatively spliced regions:

| Combination | Designated herein as |
|---|---|
| $B^-A^-V^-$ | O |
| $B^+A^-V^-$ | B |
| $B^-A^+V^-$ | A |
| $B^-A^-V^+$ | V |
| $B^+A^-V^+$ | BV |
| $B^-A^+V^+$ | AV |
| $B^+A^+V^-$ | BA |
| $B^+A^+V^+$ | BAV |

For plasma fibronectin, there can be no variants in which A and B are present.

Fibronectins play an important role in many biological systems. They have been shown to be involved in cell adhesion and migration, cell morphology, hemostasis, thrombosis and oncogenic transformation. Hynes, R.O. and K.M. Yamada, J. Cell. Biol., 95:369-377 (1982) and Hynes, R.O. Scientific American, 254:42-51 (1986).

For example, fibronectin is thought to play an important role in the cell processes involved in tissue repair, particularly wound healing. The ability to repair damaged tissue, wound healing, represents an important response to injury that is common to all complex organisms. Just as in embryonic development, this process involves cell proliferation, migration and differentiation of a number of different cell types. Fibronectin promotes cell migration in culture and is present in the embryo associated with many different cell migrations. In addition, antibodies to fibronectin or to cell surface receptors of the integrin glycoprotein family can block migration when injected into the intact embryo.

Fibronectin is expressed at high levels in healing wounds. It is derived from two sources: plasma fibronectin, which is present in the exudate from damaged blood vessels, and cellular fibronectin, which is synthesized locally in the wound tissue. Fibronectin appears to be involved in the migration <u>in vitro</u> of four major cell types that migrate into the area of the wound. Fibroblasts and epithelial cells are stimulated to migrate by fibronectin.

Presently, only plasma fibronectin is available (e.g., for therapeutic uses) in quantity. However, it is a mixture of variants and is impure and, thus, its use in treatment may be of limited value, particularly in those circumstances in which a pure form or a combination of selected variants, rather than a mixture of variants, would be more effective.

Summary of the Invention

The present invention relates to a method of producing cellular fibronectin of mammalian origin through the use of genetic engineering techniques, as well as to cellular fibronectins produced by the method. Until now, it has not been possible to produce essentially pure cellular fibronectin. The fibronectins of the present invention, however, are homogeneous cellular fibronectins which include, as desired, region B, region A or both regions, as well as the V region, if desired. Recombinant fibronectins having portions of these regions can also be produced. Thus, the fibronectins of the present invention differ from presently-available fibronectins in that the subject fibronectins are recombinant homogeneous cellular fibronectins which include region B and/or region A, alone or in combination.

In one embodiment of the method of the present invention, a recombinant full length cDNA encoding cellular fibronectin is introduced into an appropriate host cell by means of a recombinant retrovirus (or other suitable vector). The full length cDNA is expressed in the host cell, resulting in production of full length cellular fibronectin. The eight possible variants of full length rat cellular fibronectin (i.e., those including some, none or all of the three alternative splice domains designated A, B and V) have been expressed in this way, characterized, and shown, using art-recognized methods, to be biologically functional. Characterization of the recombinant cellular fibronectin produced as described herein has demonstrated that the method results in production of homogeneous populations of homodimers

(rather than the mixtures of heterodimers which naturally occur).

Brief Description of the Drawings

Figure 1 is a schematic representation of fibronectin and its variants.

Figure 2 is a schematic representation of the method of the present invention, by which recombinant full length fibronectin is produced.

Figure 3 shows the entire nucleic acid sequence encoding recombinant full-length rat fibronectin.

Figure 4 demonstrates expression of different variants of fibronectin in WEHI231 cells, which are lymphocytes which do not themselves produce fibronectin. Immunoprecipitation, using a polyclonal antibody (R61.1) which recognizes total fibronectin, was carried out, followed by reduced and nonreduced gel electrophoresis. WEHI231 cells infected with a recombinant vector containing one of the following were analyzed: pLJ, control (no fibronectin variant); O, $B^-A^-V^-$; B, $B^+A^-V^-$; A, $B^-A^+V^-$; V, $B^-A^-V^+$. Results of reduced gel electrophoresis demonstrate the presence of the fibronectin monomer and results of nonreduced gel electrophoresis demonstrate dimerization of fibronectin.

Figure 5 is a graphic representation of spreading or adhesion of melanoma cells (B16F10) on various variants and concentrations of recombinant fibronectin produced in WEHI231 cells. O, B, A and V indicate the fibronectin variants used (see Figure 4 for variant type).

Figure 6 is a photograph which demonstrates the ability of recombinant fibronectin variant A ($B^-A^+V^-$) produced in WEHI231 cells to promote reversion of Nil.8-HSV morphology to a normal morphology.

Figure 7 is a photograph which demonstrates the results of assessment of extracellular matrices of NIH 3T3 cells and incorporation of recombinant fibronectin variants B ($B^+A^-V^-$) and V ($B^-A^-V^+$) produced in WEHI231 cells into the matrices at three concentrations: -, no added fibronectin; 30 ug/ml.; 90 ug/ml.

Figure 8 is a schematic representation of the rat fibronectin structure which is composed of three repeating peptide units termed Type I, II and III, which are shown by boxes. Two alternatively spliced type III repeats, EIIIB and EIIIA, are represented by the filled squares and are indicated above the diagram. The V region is marked by the shaded box and the V25 segment is indicated with stripes, and its amino acid sequence is given below. Domains that interact with fibrin, collagen, and heparin are illustrated above. The sites that interact with cell surfaces are also shown.

Figure 9 shows the peptide inhibition of WEHI231 cell spreading on the V form of FN. The average scores of three independent experiments for each peptide are shown with their standard deviations (A). The peptide sequences are illustrated in (B). O represents the score in the absence of any peptide competitor. RGD and RGE represent peptides GRGDSP and GRGESP, respectively.

## Detailed Description of the Invention

The present invention provides a method of producing recombinant cellular fibronectin which is essentially

full length (recombinant cellular fibronectin) and of producing modified, essentially full-length recombinant cellular fibronectin. cDNA encoding recombinant cellular fibronectin has been expressed in NIH 3T3 and WEHI231 cells and tested on several cell types (CHO, Rat-1, BHK, B16 melanoma) which are standard cell types used to assay fibronectins. It has been shown, using art-recognized methods, to be produced in the form of essentially pure homogeneous homodimers and to be biologically active.

Recombinant Cellular Fibronectin

The following is a description of construction of cDNA encoding cellular fibronectin variants and of vectors useful for introduction of the cDNA into appropriate host cells, expression of the encoded cellular fibronectin in host cells containing the cDNA and characterization of the recombinant cellular fibronectin produced in this manner. Although the following describes cellular fibronectin of rat origin, it is to be understood that the same procedures can be used with cDNA encoding cellular fibronectin from other sources (e.g., human) to produce the encoded recombinant fibronectins.

Fibronectin and its variants are represented schematically in Figure 1. As shown, fibronectin includes regions which bind to various proteins or other substances (e.g., fibrin, heparin, collagen, gelatin), as well as three regions, described above, designated EIIIB, EIIIA and V. Regions EIIIB and EIIIA can be present in cellular fibronectin, but not in plasma fibronectin. Region V can be present in variants of both cellular and plasma fibronectin. As described above, there are eight

possible combinations of these three regions (eight variants) in the case of full length cellular fibronectin. cDNA encoding each of these eight variants has been produced by combining or joining cDNA fragments, each of which encodes a portion or segment of cellular fibronectin. See, Patel, R.S. et al., The EMBO J., 6:2565-2572 (1987); Schwarzbauer, J.E. et al., The EMBO J., 6:2573-2580 (1987).

The full length cDNA was introduced into appropriate cells, in which it was expressed and subsequently secreted, in the following manner, which is represented schematically in Figure 2. Figure 3 shows the entire nucleic acid sequence encoding recombinant full-length rat fibronectin. Recombinant full length cDNA encoding fibronectin was introduced into the pLJ vector, using known techniques. See, Schwarzbauer, J.E. et al., Proc. of the Natl. Acad. of Sci., USA, 84:754-758 (1987). Schwarzbauer has also modified the vector by removal of a splice site. This modifed vector is called pLJ. The characteristics of pLJ have been described in Korman, A.J. et al., Proc. of the Natl. Acad. of Sci., USA, 84:2150 (1987). This vector is capable of expressing both the gene of interest and a dominant selectable marker, such as the neo gene. The gene of interest is cloned in direct orientation into a BamHI/SmaI/SalI cloning site just distal to the 5' LTR, while the Neo gene is placed distal to an internal promoter (from SV40) which is located 3' of the cloning site. Transcription from pLJ is initiated at two sites: 1) the 5' LTR, which is responsible for expression of the gene of interest and 2) the internal SV40 promoter, which is responsible for expression of the neo gene.

The diagram at the top of Figure 2 is a representation of pLJ and the additional sequences inserted into it (e.g., full length cDNA encoding cellular fibronectin and a neomycin resistance-encoding gene, NEO-R), resulting in production of a recombinant retrovirus designated pLJ-FN. The NEO-R gene product confers resistance to the antibiotic, G418, in mammalian cells and can be used to select cells containing the recombinant vector. The resulting plasmid (pLJ-FN) was introduced into a packaging cell (e.g., Psi 2 cells), in which the cDNA was transcribed and the resulting fibronectin mRNA and NEO-R mRNA incorporated or packaged into viral particles which subsequently bud out of the cells.

If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a *cis* defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all virion proteins. Mulligan and co-workers have described retroviral genomes from which these Psi sequences have been deleted, as well as cell lines containing the mutant stably integrated into the chromosome. Mulligan, R.C., *In: Experimental Manipulation of Gene Expression*, M. Inouye (ed.), 155-173 (1983); Mann, R. *et al.*, *Cell*, *33*:153-159 (1983); Cone, R.D. and R.C. Mulligan, *Proc. of the Natl. Acad. of Sci., USA*, *81*:6349-6353 (1984).

The Psi 2 cell line described by Mulligan and co-workers was created by transfecting NIH 3T3 fibroblasts with pMOV-$\Psi^-$, which is an ecotropic Moloney murine leukemia virus (Mo-MuLV) clone. pMOV-$\Psi^-$ expresses all the viral gene products but lacks the Psi

sequence, which is necessary for encapsidation of the viral genome. pMOV-Psi$^-$ expresses an ecotropic viral envelope glycoprotein which recognizes a receptor present only on mouse (and closely related rodent) cells.

Another cell line is the Psi$^-$am line, which are Psi-2-like packaging cell lines. These Psi$^-$am cell lines contain a modified pMOV-Psi$^-$genome, in which the ecotropic envelope glycoprotein has been replaced with envelope sequences derived from the amphotropic virus 4070A. Hartley, J.W. and W.P. Rowe, Journal of Virology, 19:19-25 (1976). As a result, they are useful for production of recombinant virus with a broad mammalian host range, amphotropic host range. The retrovirus used to make the Psi-am cell line has an amphotropic host range and can be used to infect human cells. If the recombinant genome has the Psi packaging sequence, the Psi-am cell line is capable of packaging recombinant retroviral genomes into infectious retroviral particles. Cone, R. and R. Mulligan, Proc. of the Natl. Acad. Sci., USA, 81:6349-6353 (1984).

The retroviral genome has been modified by Cone and Mulligan for use as a vector capable of introducing new genes into cells. The gag, the pol and the env genes have all been removed and a DNA segment encoding the neo gene has been inserted in their place. The neo gene serves as a dominant selectable marker. The retroviral sequence which remains part of the recombinant genome includes the LTRs, the tRNA binding site and the Psi packaging site. Cepko, C. et al., Cell, 37:1053-1062 (1984). In this instance, full-length fibronectin cDNA was constructed as described and inserted into the vector, as indicated in Figure 2.

The resulting recombinant retrovirus (pLJ-FN) was used to infect appropriate recipient/host cells (e.g., NIH 3T3, WEHI231). In these cells, reverse transcription of the viral RNA results in generation of a DNA copy, which integrated into the host cell genome. Infected cells, which were G418 resistant, synthesized the encoded recombinant fibronectin variant, which was subsequently secreted into the culture medium.

Each of the eight fibronectin variants in which EIIIB, EIIIA and/or V can be present was expressed in at least one of the following two types of mammalian cell: NIH 3T3 and WEHI231. Methods for making the variants are described in detail in the Exemplification. WEHI231 cells expressing the V fibronectin variant ($B^-A^-V^+$) have been deposited, according to the terms of the Budapest Treaty, at the American Type Culture Collection (Rockville, MD) under Accession No. CRL10019. The resulting recombinant fibronectin was tested on several cell types (e.g., CHO, Rat-1, BHK, B16 melanoma cells) which are, as mentioned previously, standard cell types used to assay fibronectins. The resulting recombinant fibronectins were characterized and shown to be homodimers, rather than the heterodimers produced naturally. They have been shown, as described below, to be biologically functional. Naturally-occurring fibronectin is known to bind anti fibronectin antibodies, gelatin, and heparin; to promote adhesion of several different cell types, cytoskeletal assembly and cell migration; and to participate in reversion of tumor cells (transformed cells) to normal morphology. As described below, recombinant fibronectin produced as described herein has been shown to have these same capabilities.

Expression of recombinant fibronectin (BAV) in NIH 3T3 cells was clearly demonstrated and the results confirmed by Northern blot analyses. Two transcripts were detected from three individual clones infected with retrovirus containing the BAV form FN gene when the $neo^r$ gene was used as probe. The 11.6- and 3.1-kb messages which were observed corresponded with the sizes expected for the full-length genomic transcript from the viral LTR and subgenomic RNA from the SV-40 promoter. No band was detected in RNA isolated from parental 3T3 cells. RNA isolated from cells containing pLJ vector alone included the 3.1-kb subgenomic RNA and a minor band migrating at 3.9 kb, which corresponds with a transcript derived from the 5' viral LTR. The identity of the 11.6-kb transcript was confirmed by hybridizing the same blot with a probe derived from rat FN cDNA. This probe also detects the endogenous murine FN message (8.1 kb), which is present in rat FN expressor clones as well as in the control cell lines. The rat FN mRNA signal was ~10% of the signal for endogenous murine FN mRNA. Therefore, cDNA clones for rat FNs are readily transcribed in murine 3T3 cells under the control of the MLV-LTR promoter.

To detect and quantitate secretion of recombinant rat FNs from 3T3 cells, immunoprecipitations of supernatants harvested from [$^{35}$S]methionine-labeled cells were carried out with a mouse mAb, M9, specific for rat FN or with an anti-rat FN polyclonal serum R61. SDS-PAGE analysis of immunoprecipitates from representative clones expressing O, B, V, or BV rat FN forms. All these forms of rat FN were secreted into the medium as proteins with molecular masses around 220-250 kD. The slight differences in molecular weight are as expected from

their differences in polypeptide chain by including neither, either or both of the EIIIB and V regions. As seen most evidently in cells secreting the O form of rat FN, the endogenous mouse FN (a mixture of different forms but mostly larger than the O form) is coprecipitated with M9. This result showed that the recombinant rat FN can form dimers with the endogenous mouse FN which has been observed before for 3T3 cells expressing the COOH-terminal third of rat FNs. Schwarzbauer et al., Proc. Natl. Acad. Sci. USA 84:754-758 (1987). No endogenous FNs were precipitated by M9 from supernatants of 3T3 parental cells or clones infected with vector alone. The immunoprecipitations with the polyclonal anti-FN serum demonstrated that all these clones secreted comparable amounts of total fibronectins. Estimates from densitometry of the autoradiographs showed that the recombinant rat FNs secreted from 3T3 cell clones represent ~10% of the total FNs produced by these cells. This estimate corresponds well with the 10% rat FN transcripts compared with total fibronectin message as determined from Northern blot analysis. Thus, the chimeric rat FN mRNAs are efficiently translated and the rat fibronectins expressed in stable infected NIH 3T3 cell clones are efficiently processed, assembled into dimers and secreted into the medium. These cells also assemble the recombinant rat FNs into extracellular matrix.

Recombinant fibronectin was also expressed in WEHI231 cells. The single cell clones secreting the corresponding rat FNs were isolated by limiting dilution from the G418-resistant pools. Secretion of various forms of rat FNs was determined by immunoprecipitation

using the polyclonal antiserum R61 followed by SDS-PAGE analysis in the presence or absence of reducing agents. A major protein product migrating at 220-250 kD was immunoprecipitated from the media of [$^{35}$S] methionine-labeled cells clones expressing the O, B, A or V forms of rat FNs. The apparent molecular weights of the proteins are as expected for the various rat FN forms and also correspond with those of rat FNs expressed in NIH 3T3 cells as described above. This suggests that no major different posttranslational modifications occurred in recombinant FN synthesized in lymphoid WEHI231 cells which normally do not produce any endogenous FNs.

WEHI231 cells are lymphocytes and, thus, do not produce fibronectin. Ralph, P. Immunol. Rev., 48:107-121 (1979). Subsequent analyses demonstrated that all variants produced by WEHI231 cells bind to gelatin and heparin. For example, variants O ($B^-A^-V^-$), B ($B^+A^-V^-$), A ($B^-A^+V^-$) and V ($B^-A^-V^+$) bind both to gelatin and to heparin (as well as to a polyclonal antibody, R61.1, which recognizes total fibronectin).

All fibronectin variants produced were also shown to promote cell adhesion or spreading, in a variety of cell types. One representative experiment was performed with the mouse melanoma cell line B16F10. On control substrata coated only with bovine serum albumin (BSA), the cells either did not attach, or spready poorly. On substrata coated with a low amount of recombinant FN (in this case 2 μg/ml A form), significantly increased numbers of cells attach, but relatively few assume a well-spread morphology. When plated on higher doses of FNs (16 μg/ml A form), the majority of the cells adhered and spread well. This observation was confirmed by

quantitative measurements shown in Fig. 5 for B16F10 melanoma cells. The percentages of cells spreading are plotted as average scores for three independent experiments and the SDs were <10%. This experiment demonstrated that these four forms of recombinant FNs have similar dose-response curves in promoting B16F10 cell spreading, reaching saturating concentration at ~10 μg/ml. These results suggest that, in the basic adhesion and spreading assays with these established adherent cell lines, all forms of FN tested are equivalent.

Recombinant fibronectin variants were also shown to promote cytoskeletal organization, as assessed using antibodies against actin or vinculin. Through their transmembrane integrin receptors, extracellular FNs can induce cytoskeletal organization including organized actin bundles and focal contact formation. To examine these transmembrane effects, B16F10 melanoma cells were cultured on substrata coated with various recombinant FNs. 2 hours later, cells were fixed and stained for F actin and vinculin distribution using double-label immunofluorescence. When low concentrations of the various FNs were used, actin bundles were visualized in only a small percentage of cells while diffuse and unorganized patterns were evident for most cells. Similarly, few focal contacts were detected as determined by staining for vinculin. When plated on higher concentrations of FNs, however, extensive microfilament bundles were detected for the majority of cells and discrete focal contacts were localized at termini of actin bundles. These results indicated that recombinant FN V form was able to induce cytoskeletal organization. Similar effects were observed for the other forms of FNs obtained from WEHI231 cells (O, V, B forms).

Recombinant variants were also shown to promote reversion of Nil.8-HSV (transformed) morphology to normal cell morphology, using the method of assessing reversion described by Ali and co-workers. Ali, I. et al., Cell, 11:115-126 (1977). As shown in Figure 6, a substantial population of NIL8.HSV cells are rounded and detached from the substrate. The cells assumed a more flattened and aligned morphology upon addition of the recombinant FN A form. Similar effects were also observed for O, B, V forms of FNs. The differences in dose response among the different forms were, at most, two to threefold in different experiments. Therefore, in agreement with results obtained from basic adhesion and spreading assays using adherent cells, the ability of FN to revert the morphology of these transformed cells does not appear to reside in the EIIIB, EIIIA, or V regions.

Assessment of incorporation of recombinant fibronectin into cell matrices was also carried out and showed that all variants were incorporated and that variants $B^+$ ($B^+A^-V^-$) and $A^+$ ($B^-A^+V^-$) were incorporated somewhat more effectively than the other variants. As shown in Figure 7, recombinant rat FNs formed fibrillar networks characteristic of the usual extracellular matrix distribution of FN. Furthermore, total extracellular FN staining (with polyclonal antiserum R61) increased significantly upon addition of exogenous recombinant FNs, indicating their contribution to matrix formation. All forms of FNs incorporated into the existing matrices. However, two to threefold differences in the doses of recombinant FNs required to give a particular level of rat FN-specific fluorescence were noted. The O and V

forms required higher levels added than did the A and B forms to give equivalent staining. Minimum doses for detectable M9 staining were 10 μg/ml for B or BAV and 30 μg/ml for V. Figure 7 shows approximately equivalent incorporation of rat FN at 30 μg/ml B form and 90 μg/ml V form. These results suggest that inclusion of the EIIIA or EIIIB segments characteristic of tissue FN may enhance the ability of FN to incorporate into existing matrix.

Thus, the assays carried out, using standard techniques, resulted in the determination that the recombinant fibronectin variants are produced and are biologically functional. The following is an outline of the results obtained:

## Retroviral Expression of Fibronectin

| | | |
|---|---|---|
| 1. | Full length fibronectin variants are efficiently expressed. | |
| 2. | All forms are assembled and secreted. | |
| 3. | All forms bind to gelatin and heparin. | |
| 4. | Cell adhesion and spreading (BHK/B16/NIL8-HSV/CHO/RAT-1) | - ALL FORMS WORK WELL |
| 5. | Cytoskeletal organization. (BHK/B16) | - AT MOST 2-3X DIFFERENCE IN DOSE RESPONSE |
| 6. | Reversion to normal morphology. | - $A^+$ OR $B^+$ FORMS SLIGHTLY MORE EFFECTIVE |
| 7. | Migration. (NIL8-HSV) | - ALL FORMS WORK |
| 8. | Matrix Assembly. | - $A^+$ OR $B^+$ FORMS ARE MORE EFFECTIVE |

Modified Essentially Full Length Recombinant Fibronectins

Fibronectins having a portion of regions B, A, V or combinations of these can also be produced. For example, the first 25 amino acids of the V region (referred to as V25 segment) which are shown in Figure 8 can be selectively spliced out independently of the rest of the V region. The V25 segment is important in the selective adhesion of various cell types and is recognized by the integrin $\alpha_4\beta_1$ fibronectin receptor. $V^+$ recombinant fibronectins having the V25 segment alternatively spliced out can be produced as homodimers or heterodimers.

WEHI231 lymphoid cells interact with an alternatively spliced region of FN, specifically with the C-terminal 10 amino acids (GPEILDVPST) of the V25 segment. Only those forms of FN that contain this segment promote spreading of these cells, and this spreading can be blocked either by synthetic peptides from the V25 segment or by antibodies to the integrin $\alpha_4$ subunit. Furthermore, integrin $\alpha_4\beta_1$ binds specifically to the V25 peptide coupled to Sepharose.

Thus, integrin $\alpha_4\beta_1$ is an FN receptor distinct from the $\alpha_5\beta_1$ integrin receptor, which recognizes the RGDS site in FN (Pytela et al., Cell 40:191-198 (1985); Pytela et al., Science 231:1559-1562 (1986); Argraves et al., J. Cell Bio. 105:1183-1190 (1987); and Wayner et al., J. Cell Bio. 107:1881-1891 (1988)) and another integrin $\alpha_3\beta_1$, which also binds to FN at an unknown site (Takada et al., J. Cell Biochem. 37:385-393 (1988); Wayner, E.A. and W.G. Carter, J. Cell Bio. 105:1873-1884 (1987); and Wayner et al., Ibid. (1988). While $\alpha_3\beta_1$ and $\alpha_5\beta_1$ are prevalent on cultured fibroblastic cells (Helmer et al.,

J. Bio. Chem. 262:3300-3309 (1987); Wayner, E.A. and W.G. Carter, Ibid. (1987); and Wayner et al., Ibid. (1988)), and the adhesion of such cells to FN is frequently blocked by RGDS-containing peptides (Pierschbacher, M.D. and E. Ruoslahti, Nature 309:30-33 (1984); and Pierschbacher, M.D. and E. Ruoslahti, Proc. Natl. Acad. Sci. USA 81:5985-5988 (1987)), $\alpha_4\beta_1$ is expressed predominantly on lymphoid and myeloid cells (Helmer et al., J. Bio. Chem. 262:3300-3309 (1987); Helmer et al., Ibid. (1987); Wayner et al., Ibid. (1989)).

The V25 segment can be selectively spliced out independently of the rest of the V region in mammals (Schwarzbauer et al., Cell 35:421-431 (1983); Kornblihtt et al., Nucl. Acids Res. 12:5853-5868 (1985); Sekiguchi et al., Biochemistry 25:4936-4941 (1986)) and a 44 amino acid segment (V44) that includes V25 can be similarly spliced out in chickens (Norton, P.A. and R.O. Hynes, Mol. Cell. Biol. 7:4297-4307 (1987); french-Constant, C. and R.O. Hynes, Development 106:375-388 (1989)). The sequences of these segments are well conserved; the V25 segment is identical in humans, rats, and cows (Peterson et al., "Primary structure of fibronectin" In Fibronectin, D.F. Mosher, ed. (San Diego, Academis Press) (1989); and Hynes, R.O., Fibronectins (New York:Springer-Verlag) (1989)), and the segment corresponding to the V10 peptide in chickens contains 60% identical and 80% homologous residues (Norton, P.A. and R.O. Hynes, Ibid. (1987)). This conservation is consistent with an important role for this segment, and Humphries et al., J. Bio. Chem. 262:6886-6892 (1987) have previously reported that peptides corresponding to this segment will inhibit

adhesion of murine B16F10 melanoma cells when soluble and promote their adhesion when bound to a surface. They showed also that V25 peptides (CS-1) coupled to proteins will promote neurite outgrowth of chicken peripheral neurons (Humphries et al., J. Cell Bio. 106:1289-1297 (1988)) and attachment and migration of neural crest cells (Dufour et al., EMBO J., 7:2661-2671 (1988)).

However, most of the experiments on mammals do not test for selective exclusion of the V25 segment, which can be omitted independently of other parts of the V region in mammals. Given the difficulty of assaying for the absence of this segment, it is not yet possible to say whether or not V25-negative forms of FN may be found in specific locations. The absence of the V segment from 50% of plasma FN subunits is of potential relevance given the fact that many circulating blood cells express $\alpha_4\beta_1$ (Helmer et al., J. Bio. Chem. 262:3300-3309 (1987); Helmer et al., J. Bio. Chem. 262:11478-11485 (1987); Wayner et al., J. Cell Biol. 109:1321-1300 (1989)). Since plasma FN appears to be a heterodimer of $V^+$ and $V^-$ subunits (Schwarzbauer et al., J. Cell Biol., 109:3445-3453 (1989) each molecule contains only a single binding site for $\alpha_4\beta_1$. This appears to be insufficient for high affinity binding to cell surfaces since FN is not found as a surface component of circulating blood cells. If, as appears to be the case, matrix FN is largely $V^+$, it should have a higher avidity for binding of cells bearing $\alpha_4\beta_1$ integrin. This could play a role in the adhesion of various blood cells to exposed extracellular matrix, such as endothelial basement membrane. In this context, it is interesting to note that $\alpha_4\beta_1$ has recently been reported

to be involved in homing of lymphocytes to Peyer's patch high endothelial venules (Holzmann et al., Cell 56:37-46 (1989)).

In the integrin $\beta_1$ subfamily there are three FN receptors: $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_3\beta_1$ specific, respectively, for the RGDS site, 10 amino acids in the V25 segment, and an unknown site. Other integrins are also known to interact with FN, including $\alpha_{IIb}\beta_3$ (GPIIb/IIa), which binds to the RGDS site (Gardner and Hynes, Cell 42: 439-448 (1985); Pytela et al., Science 231:1559-1562 (1986); D'Souza et al., J. Bio. Chem. 263:3942-3951 (1987)) and $\alpha_v\beta_x$, which probably also binds to the RGDS site (Cheresh et al., Cell 57:59-69 (1989)).

Production of Recombinant Cellular Fibronectin

Fibronectin of the present invention can be produced, as described previously, using an appropriate vector containing cDNA encoding full-length recombinant cellular fibronectin, which is introduced into and expressed by an appropriate host cell. The DNA encoding the cellular fibronectin can be cDNA or DNA, synthesized by known methods, which has the same nucleotide sequence as the cDNA or a functional equivalent thereof (i.e., one which encodes a product having the same characteristics and exhibiting the same functions as the recombinant cellular fibronectin described herein). Appropriate host cells include, but are not limited to, NIH 3T3 and WEHI231 cells; other cells in which the complex cellular fibronectin can be produced and properly processed can also be used.

Uses of Recombinant Fibronectin Produced by the Present Method

Fibronectin produced by the process of the present invention has several therapeutic and clinical uses, and can be used wherever fibronectin is naturally utilized in the body. For example, fibronectin plays an important role in the cell migration associated with wound healing and tissue repair in general. Immunolocalization studies have shown that abundant fibronectin is present in healing wounds. In situ hybridization studies have shown that cellular fibronectin synthesized locally in wound tissue contains both the B and the A segments or regions.

Fibronectin can also be used to promote nerve regeneration in some cases. For example, while not strictly cell migration, the outgrowth of neurites from neurons involves many of the same principles and similar mechanisms. There is considerable evidence that some neurons will respond to fibronectin by extending neurites. It has been shown that fibronectin promotes outgrowth of neurites from aggregates of 7-day chick neural retinal cells. Akers, R.M. et al., Dev. Biol., 86:179-188 (1981). Fibronectin has been shown to promote neurite growth in chick ganglion cells (Carbonetto, S.T. et al., J. Neurosci., 3:2324-2335 (1983) and in human ganglion cells. (Baron-Van Evercooren, A. et al., J. Neurosci. Res., 8:179-183 (1982)).

Fibronectin also plays a role in hemostasis and thrombosis and can be used in the treatment of blood or clotting disorders. Thrombotic diseases are major killers, and the ability to control thrombosis would be a valuable therapeutic tool in treating them. Monoclonal

antibodies raised against fibronectin, or specific peptides, could be used to intervene in the interactions between the ligand and its receptors. For example, fibronectin interacts with fibrin, becomes incorporated into clots, and is crosslinked to the fibrin by factor XIIIa transglutaminase. Therefore, as the clot is formed from platelets and fibrin, fibronectin becomes an integral part of it. Intervening in this reaction by preventing fibronectin from performing its usual role provides a method of preventing dangerous blood clots. It has been shown that soon after wounding, fibronectin and fibrin appear in the area of a wound. These proteins then serve as a substrate for adhesion and migration of the cells repairing the defect and, in most cases, subsequently disappear. Fibronectin also plays a role in removal of debris by various cell types.

When a wound is made in the skin, several wound healing processes ensue. The epidermal cells migrate in to cover the wound. Concurrently, beneath the healing epidermal layer, granulation tissue forms and eventually neovascularization follows. These three processes involve different cell types and are best considered separately.

The first detectable event is the formation of a fibrin-fibronectin clot in the area of the wound. Grinnell, F. et al., J. Invest. Dermatol., 76:181-189 (1981); Clark, R.A. et al., J. Invest. Dermatol., 79:264-269 (1982); Repesh, L.A. et al., J. Histochem. Cytochem., 30:351-358 (1982). There is a large increase in fibronectin soon after wounding and the epidermal cells migrate beneath the main clot on the fibronectin-

fibrin matrix. After wound epithelialization is complete, the thickened basement membrane becomes thinner again and the fibrin and fibronectin fall to low levels and are replaced by laminin and type IV collagen typical of the normal BM. In an experiment involving wounding of rat skin implanted in mice, Clark and co-workers were able to distinguish locally produced cellular fibronectin (rat) from deposited plasma fibronectin (mouse). Clark, R.A. et al., J. Invest. Dermatol., 80:26s-30s (1983). They found that the early, provisional matrix consisted largely of plasma fibronectin which was gradually replaced by cellular fibronectin during epidermal wound healing.

Donaldson and Mahan have shown directly that fibronectin and fibrin promote epidermal migration, using implants placed in wounds in new skin. Donaldson, D.J. and J.T. Mahan, J. Cell Sci., 62:117-127 (1983) and Donaldson, D.J. and J.T. Mahan, Cell Tissue Res., 235:221-224 (1984). Coating of the implants with fibronectin or fibrin promoted migration in a dose-dependent fashion and this was specifically inhibited by antibodies to these two proteins.

Fibronectin can be used to treat corneal lesions. The cornea of the eye is a relatively simple, nonvascularized system. The cornea consists of a layer of epithelial cells on a basement membrane, beneath which lies a thick stromal layer composed largely of collagen with a few keratocytes. Beneath the stroma is a specialized basement membrane (Déscemet's membrane) with a layer of endothelial cells attached to it. Fibronectin is believed to be involved in the migration of the

endothelial cells and stromal cells during development, but in the mature cornea the only significant concentration of fibronectin is in Descemet's membrane; the basement membrane of the corneal epithelium has little fibronectin. Kurkinen, M. et al., Dev. Biol., 69:589-600 (1979) and Cintron, C. et al., Curr. Eye Res., 3:489-499 (1984).

Within a few hours after a scrape wound which removes the epithelial layer, fibronectin and fibrin appear on the surface of the cornea. Between one and two days after wounding, the corneal epithelium grows back over this fibronectin and fibrin-coated surface and the fibronectin and fibrin disappear over the next several days. Fibronectin also appears in significant amounts within the stroma which normally contains only small amounts.

This pattern of appearance of fibronectin and fibrin indicates that these proteins form the substrate promoting migration of the healing epithelium. Nishida and co-workers have cultured blocks of cornea in vitro and shown that the epithelial layer migrates over the cut stromal surface. Nishida, T. et al., Jpn. J. Ophthalmol., 26:410-415 (1982) and Nishida, T. et al., Jpn. J. Ophthalmol., 26:416-424 (1982). The migrating epithelium is underlain by a layer of fibronectin. Addition of autologous serum or, more significantly, purified plasma fibronectin to the cultures accelerated the epithelial migration and anti-fibronectin antisera inhibited it. Thus, fibronectin can be used in the treatment of corneal epithelial wounds.

Fibronectin has therapeutic value in promoting corneal epithelial wound healing and in leading to curing of persistent corneal ulcers. Fibronectin can be used to prepare eye drops, for example. Treatment with these eye drops leads to accelerated healing of corneal ulcers and other defects. It has been shown that the protease inhibitor, aprotinin, has dramatic therapeutic effects on the healing of corneal lesions. Thus, combinations of fibronectin and protease inhibitors may prove particularly efficacious. Past efforts in improving wound healing have made use of plasma fibronectin, which, as mentioned, is a mixture of types. Homogeneous cellular fibronectin might be more effective in promoting wound healing (e.g., because of the presence of B and/or A regions).

Fibronectin treatment may be useful in treatment of periodontal disease, which is characterized by failure of attachment of gingival tissue to the tooth roots. Studies *in vitro* have shown that attachment and migration of gingival fibroblasts and periodontal ligamentum cells on teeth and bone fragments is promoted by citric acid demineralization to expose collagen and by fibronectin treatment. Terranova, V.P. and G.R. Martin, *J. Periodont. Res.*, 17:530-533 (1982); Fernyhough, W. and R.C. Page, *J. Periodontol.*, 54:133-140 (1983); Arisawa, Y. and Y. Abiko, *Gen. Pharmacol.*, 15:293-239 (1984); Terranova, V.P. *et al.*, *J. Periodontol.*, 58:247-257 (1987); Terranova, V.P. *et al.*, *J. Periodont. Res.*, 22:248-251 (1987). The fibroblasts attach and synthesize an extensive extracellular matrix attached to the tooth fragments. These results suggest that fibronectin

promotes attachment of gingival tissue to tooth roots *in vivo* and this appears to be the case. When tooth roots are surgically exposed and planed, connective tissue attachment during healing is significantly promoted by treatments with citric acid and fibronectin. Thus, in wound healing the fibronectin, or even conjugated peptides can be used to promote cell adhesion and migration on the wound bed. In the case of fibrosis, the accumulation of fibronectin and other matrix molecules, and the cells that produce them must be controlled, for example, by intervention in the stimulatory events between cells or in the biosynthesis of fibronectin. It is also possible to interfere with fibronectin function by the use of antibodies or peptides.

The quantity of the present fibronectin to be administered will be determined on an individual basis, and will be based at least in part on consideration of the severity of the symptoms to be treated and the result sought.

The agent or drug containing fibronectin can be administered by subcutaneous or other form of injection, intravenously, parenterally, transdermally or topically. The form in which it will be administered will depend upon the route by which it is administered.

A fibronectin composition of the present invention can optionally include other components. The components included in a particular composition are determined primarily by the manner in which the composition is to be administered. For example, a composition to be applied topically can include, in addition to fibronectin, or a derivative thereof, a binder (e.g., carboxymethyl

cellulose, gelatin), a protease inhibitor, (e.g., aprotinin), a filler (e.g., lactase) or an emulsifier. A composition to be administered dropwise (e.g., as eyedrops) may contain a liquid carrier (e.g., saline).

In general, a composition of the present invention to be applied to a skin wound, for example, would be applied directly to the wound for a period of time necessary to induce healing. More than one application may be necessary. The dosage, or concentration of fibronectin, in the composition will also vary on an individual basis and be determined by the type and severity of the symptoms to be treated.

The invention will be further illustrated by the following non-limiting Exemplification.

## EXEMPLIFICATION

### Cell Cultures

NIH 3T3 and Psi 2 cells were grown in Dulbecco's modified Eagle's medium (DME) plus 10% calf serum (CS, Gibco Laboratories, Grand Island, NY). Mouse B lymphocyte WEHI231 cells (Ralph, P. (1979), Immunol. Rev. 48:107-121) were kindly provided by D. Schatz (Whitehead Institute, MIT) and grown in RPMI 1640 medium plus 10% fetal calf serum (FCS, Gibco Laboratories). NRK, Ratl and Ni18.HSV cells were cultured in DME with 5% FCS. BHK cells were kindly provided by F. Grinnell (University of Texas, Dallas) and maintained in DME plus 10% FCS. Murine melanoma B16F10 cells were generous gifts of I.J. Fidler (M.D. Anderson Hospital, Houston) and cultured as described (Fidler, I.J., (1974), Cancer Res. 34:1074-1078).

Plasmid Construction

Retroviral vectors containing the 3' third of rat FN cDNA including or excluding EIIIA or V have been described previously (Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). Genomic clones were isolated from a rat genomic library in EMBL3B (Tamkun et al., (1984) Proc. Natl. Acad. Sci., USA 81:5140-5144) using segments from the 5' end of λrFN2 and subsequently the 5' ends of successive clones. The segments were subcloned in pGEM vectors (Promega Biotec), checked for repetitive sequences and used to screen lambda plaques by standard methods (Maniatis et al., (1982) Molecular Cloning Manual, Cold Spring Harbor Laboratory, Gold Spring Harbor, NY). Clones were analyzed by restriction enzyme mapping and Southern blotting and suitable fragments were subcloned into pGEM vectors for further analysis.

Genomic fragments were subcloned into a murine retroviral vector, pLJ, which is a derivative of DOL (Korman et al., (1987) Proc. Natl. Acad. Sci., USA 84:2150-2154) from which the 5' splice site has been deleted. As described elsewhere (Schwarzbauer et al., 1987) genomic fragments subcloned into such retroviral vectors are accurately spliced during generation of recombinant retrovirus. Cells derived by infection with these viruses therefore contain cDNAs derived from the genomic clones and these cDNAs can be recovered by fusion rescue. cDNA clones covering the 5' 2 kb of rat fibronectin encoding nine type I repeats and two type II repeats were prepared in this way.

Clones λrFN2 to λrFN5 cover the central part of the gene which includes all the type-III repeats (Figure 3).

cDNA clones isolated from a rat liver λgt11 library cover type-III repeats 9-15 (Schwarzbauer et al., (1983) Cell 35:421-431). cDNA clones covering type-III repeats 1-9 and the two alternatively spliced EIII repeats were obtained by passage of genomic clones through retroviral vectors.

Overlapping cDNA clones covering the 5' regions of the gene were generated from the respective genomic clones, λrFN-3, λrFN-5, λrFN-8, and λrFN-9 (Patel, R.S. et al., (1987), EMBO J. 6:2565-2572; Schwarzbauer, J.E. et al., (1987), EMBO J. 6:2673-2580), using a fusion rescue method as outlined before (Schwarzbauer, J.E. et al., (1987), EMBO J. 6:2673-2580; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). The expression vector, pDOP, was constructed from pMSV-gpt (Mann, R. et al., (1983) Cell 33:153-159) by replacement of the sequences from the Kpn I site at the 3' end of the 5' long terminal repeat to the Xho I site (Mann, R. et al., (1983) Cell 33:153-159) with those from a murine leukemia virus-based vector (DO1; (Korman, A.J. et al., (1987) Proc. Natl. Acad. Sci., USA). pDOP contains a unique BamHI cloning site followed by a fragment of pBR322, the simian virus 40 (SV40) origin and early promoter, and the $neo^r$ gene (Cepko, C.L. et al., (1984) Cell 37:1053-1062; Korman, A.J. et al., (1987) Proc. Natl. Acad. Sci., USA). The $neo^r$ gene product confers resistance to the antibiotic, G418, in mammalian cells (Davies, J. and A. Jimenez (1980), Am. J. Trop. Med. Hyg. 29:Suppl. 5, 1089-1092). Thé polyoma virus early region increases the plasmid copy number after transfection into Ψ2 cells.

FN cDNAs were isolated from a rat liver λgt11 library (Schwarzbauer, J.E. et al., (1983) Cell 35:421-431). BamHI and Bcl I linkers were added to the 5' and 3' ends, respectively, of an EcoRI partial-Sac II fragment of λrlf 3 containing the 3'-terminal 2400 bases of coding sequence including the 360-base variable segment (V120) plus 169 bases of the 3'-untranslated region. A 110-base-pair BamHI-Bgl II fragment from pPTHm127 (Hellerman, J.G. et al., (1984) Proc. Natl. Acad. Sci., USA 81:5340-5344) containing the 5'-untranslated and "prepro" coding sequences of parathyroid hormone was then ligated to the 5' end of the linkered FN cDNA. The hybrid cDNA was inserted into the BamHI site of pDOP. The sequence across the prepro-FN junction was confirmed (Maxam, A. and W. Gilbert (1977), Proc. Natl. Acad. Sci., USA 74:560-569). cDNAs containing the 285-base difference sequence (V95) or no additional sequences (V0) were constructed by replacing the 360-base sequence with the corresponding fragments from λrlf 4 and λrlf 6, respectively (Schwarzbauer, J.E. et al. (1983) Cell 35:421-431).

FN cDNA containing the EIII segment was obtained by passage through Ψ2 cells of a retroviral vector, DOL (Korman, A.J. et al. (1987) Proc. Natl. Acad. Sci., USA), carrying an 8-kilobase (kb) EcoRI-BamHI fragment of the FN genomic clone λrFN 2 (Tamkun, J.W. et al. (1984) Proc. Natl. Acad. Sci., USA 81:5140-5144). After infection of 3T3 cells with recombinant virus, the G418-resistant ($G418^r$) cells were fused with COS cells to induce replication from the SV40 origin, and the provirus was rescued (Cepko, C.L. et al. (1984) Cell 33:153-159). Recovered cDNA copies of this region of the FN gene were

sequenced (Maxam, A. and W. Gilbert (1977) Proc. Natl. Acad. Sci., USA 74:560-569), and all splice junctions were correct.

These cDNA clones, including or excluding EIIIB, were recombined, using unique restriction sites, with the existing 3' cDNAs to give full rise to full length cDNAs encoding rat FN. All possible combinations of EIIIB, EIIIA and V were made and the constructs confirmed by restriction mapping. These full-length cDNAs extend from a Bal I site (TGGCCA) 50 nucleotides upstream of the initiator codon of rat FN (Patel, R.S. et al., (1987), EMBO J. 6:2565-2572) to a Sac II site (CCGCGG) in the 3' untranslated region (Schwarzbauer, J.E. et al., (1983), Cell 35:421-431; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758; Patel, R.S. et al., (1987), EMBO J. 6:2565-2572). They include the entire coding region of the various forms of rat FN and the 3' untranslated region included in the earlier retroviral constructs (Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758). Thus, these constructs include the natural signal and propeptide segments of rat FN to allow normal secretion and processing. The full length clones were inserted into the retroviral expression vector pLJ to generate pLJ-FN plasmids. pLJ is a derivative of pDOL (Korman, A.J. et al., (1987), Proc. Natl. Acad. Sci. USA 84:2150-2154; Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758), from which the 5' splice site has been completely removed, and was generated and provided by J. Schwarzbauer. This was accomplished by restriction enzyme digestion of AGCTGGCCA (plural) with Alu I and Bal I and religation which deletes four bases (CTGG) to produce sequence AGCCA. The

deletion was confirmed by sequencing. cDNAs to be expressed are inserted at a cloning site downstream of the 5' MLV LTR. After the cloning site is an SV-40 origin/promoter/enhancer segment and the neomycin resistance ($neo^r$) gene driven by the SV-40 early promoter and a pBR322 origin of replication.

Transfection of Psi 2 Cells and Infection of NIH 3T3 and WEHI231 Cells

Establishment of virus-producing Psi2 cells and infection of NIH 3T3 and WEHI231 cells were performed. Schwarzbauer, J.E. et al., (1987), Proc. Natl. Acad. Sci. USA 84:754-758; Landau, N.R. et al., (1987), Mol. Cell. Biol. 7:3237-3243. 3T3 and Ψ2 cells were grown in medium plus 10% (vol/vol) calf serum. COS cells (Gluzman, Y. (1981) Cell 23:174-182) were maintained in 10% (vol/vol) fetal calf serum. DNA transfections were performed using calcium phosphate precipitation (Graham, R. and A. Van der Eb (1973) Virology 52:456-457; Parker, B.A. and G.R. Stark (1979) J. Virol. 31:360-369). Twenty hours after glycerol shock, Ψ2 medium containing transiently produced recombinant virus was removed and filtered, and 1 ml of this virus stock was used to infect 3T3 cells in the presence of polybrene at 8 μg/ml. Several days later cells were cultured in medium containing G418 at 0.5 mg/ml. The number of $G418^r$ 3T3 colonies obtained ranged from 50 to several hundred; a subset of these was isolated and expanded for further analysis. In addition, $G418^r$ Ψ2 clones were tested for virus production. Viral titers for these clones ranged from $10^3$ to $10^5$ $G418^r$ colony-forming units/ml of supernatant. The following modifications were performed. After infection, NIH 3T3

cells were selected for $neo^r$ expression in G418 (Gibco Laboratories) at a concentration of 0.5 mg/ml. A subset of G418-resistant clones was then isolated and clones were expanded for further analysis. Infected WEHI231 cells were selected with G418 at a concentration of 3 mg/ml. The selected pool of cells was then cloned by limiting dilution. Single cell clones that produced the highest amounts of recombinant FNs, as determined by immunoprecipitations, were expanded for further analysis.

Northern Blot Analysis

Total RNA was isolated from NIH 3T3 cell clones by guanidinium thiocyanate extraction followed by centrifugation through CsCl as described by Chirgwin, J.M. et al., (1979), Biochemistry 18:5194-5199). 20 μg of RNA were electrophoresed in 0.8% agarose gels containing 1.1 M formaldehyde as described (Lehrach, H. et al., (1977), Biochemistry 16:4743-4751). After electrophoresis, gels were stained with ethidium bromide to visualize the position of 28S-18S ribosomal RNA and the relative RNA content in each lane. The RNA was then transferred to Zeta-Probe blotting membranes (Bio-Rad Laboratories, Richmond, CA), which were processed using protocols recommended by the manufacturer. Duplicate RNA samples were used for hybridizations with probes corresponding to rat FN cDNA or the $neo^r$ gene. The probes were labeled with $^{32}P$ by random priming (Feinberg, A. and B. Vogelstein, (1984), Anal. Biochem. 132:6-13).

Metabolic Labeling, Immunoprecipitation, Gelatin and Heparin Binding Assays, and SDS-PAGE

Cells were labeled for 20-24 h with media containing a reduced amount of unlabeled methionine (10% of that in

normal media) and 25 μCi/ml [$^{35}$S]methionine (Tran$^{35}$S-label, ICN Radiochemicals, Irvine, CA). Conditioned media were immunoprecipitated using either a rabbit anti-rat FN serum R61 and goat anti-rabbit IgG or a mouse monoclonal anti-rat FN M9 (a generous gift of M. Chiquet) and goat anti-mouse IgG, as described (Choi, M. and R.O. Hynes, (1979), J. Biol. Chem. 254:12050-12055). Immunoprecipitates were analyzed either with or without reduction by electrophoresis through SDS-PAGE followed by fluorography. Direct binding of FNs in the conditioned media to gelatin-coupled Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was carried out as described. The bound materials were also eluted by 4 M urea in PBS and used directly for binding assays with heparin-coupled Sepharose (Pharmacia Fine Chemicals) as described by Price, J. and R.O. Hynes, (1985), J. Neurosci. 5:2205-2211.

Purification of Recombinant Rat FNs

Recombinant FNs produced from expressor WEHI231 cell clones were purified by affinity-chromatography using a gelatin-coupled Sepharose column as described by Engvall, E. and E. Ruoslahti, (1977), Int. J. Cancer 20:201-205. Briefly, the expressing clones were grown to saturation in 3 liters of growth medium. The cells were then washed with PBS and resuspended in 10 liters of RPMI 1640 plus 5% FCS that had been passed through gelatin-Sepharose 4B to deplete FN in the serum. The cells were incubated further for 3 d and the conditioned media were concentrated and subsequently loaded onto a

gelatin-Sepharose 4B column. Recombinant FNs were eluted using 4 M urea in CAPS buffer (10 mM CAPS (cyclohexylaminopropane sulfonic acid), 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, pH 11). The peak fractions as determined by UV absorption at 280 nm were pooled and dialyzed against CAPS buffer to remove urea. The final concentrations were determined again by UV absorption and also confirmed by comparison with protein standards on SDS-PAGE followed by Coomassie blue staining. Typical yields were 3-5 mg of purified recombinant FN from 10 l of culture supernatant.

Cell Spreading Assay

The biological activities of recombinant FNs were determined by a quantitative cell spreading assay modified from that described by Yamada, K.M. and D.W. Kennedy, (1984), J. Cell. Biol. 80:492-498 and Obara, M. et al. (1988), Cell 53:649-657. Restricted areas of tissue culture plates were incubated with 25 $\mu$l of serial dilutions of recombinant FNs for 2 h at room temperature, followed by incubation with 2 mg/ml heat-treated bovine serum albumin (BSA) (10 min at 80°C) in PBS for 2 hr at 37°C and extensive washing with PBS. Adherent cell lines were harvested by brief trypsinization and then washed with PBS containing 0.5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The cells were added to coated plates at $10^5$/ml in growth media without serum. Suspension WEHI231 cells were washed with PBS and added at 2 x $10^5$/ml. After 2 hr of incubation at 37°C, the plates were washed with PBS and fixed in 3.7%

formaldehyde in PBS. Percent cell spreading was then determined by counting three random fields (200-300 cells) using a Nikon inverted phase-contrast microscope.

Other FN Functional Assays

The effect of various recombinant FNs on Nil8.HSV cells was examined as described by Ali, I.U. et al. (1977), Cell 11:115-126. Two ml of growth medium containing 2 x $10^5$ cells were seeded in 35mm dishes. After 48 h, FNs were added in 100 µl PBS to give the desired concentration. Photographs were taken on the Nikon inverted phase-contrast microscope 24 h later.

To examine their effects on cytoskeletal organization, recombinant FNs were used to coat coverslips at various concentrations. Murine melanoma B16F10 cells or fibroblastic BHK cells were then plated onto the coated coverslips in the absence of serum. 2 h later, the distribution of actin bundles and vinculin was visualized by immunofluorescence. Briefly, cells were rinsed twice in PBS and fixed for 15 min in a freshly prepared 4% solution of paraformaldehyde (Fluka Chemical Co., Bern, Switzerland) in PBS, rinsed and permeabilized with 0.5% NP-40 in PBS for 15 min. Cells were stained with mouse mAb against vinculin (Sigma Chemical Co.) in 10% normal goat serum in PBS for 30 min at 37°C. After three washes with PBS, the second antibody mixture (rhodamine-conjugated goat anti-mouse IgG and fluorescein-conjugated phalloidin in 10% normal goat serum in PBS, Cappel Laboratories, Cochranville, PA) was added and incubated for 30 min at 37°C. After three washes, coverslips were mounted in Gelvatol and examined using a Zeiss Axiophot

microscope (Carl Zeiss, Inc., Thornwood, NY) and photographed using Kodak Tri-X film (Eastman Kodak Co., Rochester, NY).

Cell migration assay was carried out using a Micro Chemotaxis Chamber (Neuro Probe Inc., Cabin John, MD) as described by McCarthy, J.B. and L.T. Furcht (1984), J. Cell Biol. 7:4297-4307. Incorporation of recombinant rat FNs into the extracellular matrix of NIH 3T3 cells was determined by indirect immunofluorescence as described above except that mouse monoclonal M9 and rabbit antiserum R61 were used as the primary antibody, and fluorescein-conjugated goat anti-mouse IgG and rhodamine-conjugated goat anti-rabbit IgG were used as the second antibody.

Antibodies and Peptides

Rabbit polyclonal antisera specific for integrin subunits $\beta_1$, $\alpha_3$, and $\alpha_5$ were raised against their cytoplasmic domains as described previously (Marcantonio, E.E. and R.O. Hynes, (1988), J. Cell. Biol. 106:1765-1772; Hynes, R.O. et al., (1989), Fibronectins New York: Springer-Verlag). A rat monoclonal antibody R1-2 against mouse integrin $\alpha_4$ was kindly provided by Drs. Holzmann and Weissman (Stanford University). This antibody was raised against the $\alpha_4$ subunit as a part of the murine Peyer's patch-specific lymphocyte homing receptor (Holzmann, B. et al. (1989), Cell 56:37-46). For immunoprecipitation R1-2 was covalently coupled to CNBr-activated Sepharose 4B following standard procedures (Pharmacia Biochemicals Co., Piscataway, NJ). Rat monoclonal antibody against mouse IgM and purified rat

IgG were purchased from Accurate Chemical and Scientific Co., Westbury, NY.

The V25, V14, V10a, V10b and V10 peptides are derived from the sequences in the alternatively spliced V segment of rat FN (see Figures 8 and 9). The V10/1 and V10/2 peptides are two scrambled versions of the V10 peptide containing the same amino acids but in a different order. These peptides and GRGDSP and GRGESP were synthesized using an Applied Biosystems 430A peptide synthesizer by solid-phase t-boc chemistry. Peptides were cleaved and deprotected using trifluoro-methanesulfonic acid and were purified by reverse phase HPLC chromatography on a Vydac C18 semi-preparative column (Rainin Instruments, Woburn, MA) eluted with a 0%-60% acetonitrile gradient in 0.1% TFA.

Peptide Inhibition and Antibody Blocking Assays

For peptide inhibition assays, tissue culture dishes were coated with 60 μg/ml V form of FN. Purified peptides were added with the cells at the time of plating onto the coated dishes. For antibody blocking experiments, cells were preincubated with media containing various amounts of antibodies for 30 min at 4°C before added to the coated dishes in the continued presence of the antibodies. At least three independent experiments were performed under each condition, and the average scores are presented with standard deviations.

Cell Surface Iodination and Immunoprecipitation

Cell surface labeling with $Na^{125}I$ (New England Nuclear/DuPont, Boston, MA) and lactoperoxidase (Sigma,

St. Louis, MO) of NIH 3T3 cells was as described previously (Hynes, R.O., (1973), Proc. Natl. Acad. Sci., USA 70:3170-3174). For WEHI231 cells, a modified method was employed: $2 \times 10^8$ cells were washed with $PBS^+$ (PBS plus 1 mM $CaCl_2$ and 1 mM $MgCl_2$) and resuspended in 1 ml of $PBS^+$ containing 10 mM D-glucose (EM Science, Cherry Hill, NJ). Two millicuries of $Na^{125}I$ and a mixture of lactoperoxidase and glucose oxidase were then added to initiate the reaction (final concentrations: 20 μg/ml and 0.1 U/ml, respectively; Sigma). The labeling was allowed to continue for 10 min at room temperature with occasional rocking. Cells were then washed twice with $PBS^+$ containing 150 mM NaI and three more times with $PBS^+$.

Iodinated cells were extracted with 0.5% NP-40, and immunoprecipitation was performed as described previously (Marcantonio, E.E. and R.O. Hynes, (1988), J. Cell. Biol. 106:1765-1772). To detect integrin $\alpha_4$, extracts were immunoprecipitated using Sepharose coupled with the rat monoclonal antibody R1-2, followed by direct recovery by boiling for 3 min in the sample buffer (2% SDS, 100 mM Tris-HCl [pH 6.8], 10 mM EDTA, 10% glycerol and bromophenol blue). For some experiments, integrin complexes extracted from labeled WEHI231 cells were first dissociated by heating at 100°C for 2 min in 1% SDS. After cooling, a 5-fold excess of Triton X-100 was added, and the extracts were precipitated with antiserum against $\beta_1$ as described above. SDS-PAGE was performed by the method of Laemmli, U.K. (1970), Nature 227 680-685. Separation gels were 7% acrylamide with a 3% stacking gel.

Direct Binding Assay and Affinity Chromatography Using V25-Sepharose

The V25 peptide was covalently coupled to CNBr-activated Sepharose 4B at 2 mg peptide per ml beads according to instructions provided by the manufacturer (Pharmacia Biochemicals Co., Piscataway, NJ). For direct binding assays, iodinated WEHI231 cells were extracted using 200 mM n-octyl-$\beta$-D-glucopyranoside in 50 mM Tris[pH 7.5], 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (TBMMC) for 30 min on ice. These extracts were incubated with V25-Sepharose beads for 3 hr at 4°C, followed by washing in 50 mM n-octyl-$\beta$-D-glucopyranoside in TBMMC (washing buffer) four times. The bound material was then eluted with the sample buffer and subjected to SDS-PAGE as described above.

For affinity chromatography, 2 ml of extracts from $^{125}I$-labeled WEHI231 cells were loaded onto 1 ml (packed volume) V25-Sepharose by incubation at 4°C for 1 hr, followed by washing with 30 ml of washing buffer. Columns were eluted with 2 ml of washing buffer containing 1 mg/ml V14 peptide, followed by 1 ml of washing buffer, 2 ml of washing buffer containing 1 mg/ml V10 peptide, and 2 ml of washing buffer. Fractions of 400 $\mu$l were collected, and 40 $\mu$l of each fraction were analyzed by SDS-PAGE. Peak fractions eluted by the V14 and V10 peptides were pooled, immunoprecipitated and analyzed by SDS-PAGE, along with the starting material, as described above.

Integrin Receptor Binding

Peptide inhibition experiments were performed to define the active site in the V segment for interaction with WEHI231 cells and thus to facilitate the identification of a receptor from these cells. Its effect on WEHI231 cell spreading was examined by including the soluble peptide in spreading assays on saturating amounts of the V form of FN (60 μg/ml). The V25 peptide inhibited the spreading of WEHI231 cells in a dose-dependent manner. At a concentration of 300 μg/ml, the inhibition was greater than 80%. By contrast, the peptide had little effect on the spreading of NIH 3T3 cells on FN, demonstrating its specificity. The effect of peptides GRGDSP and GRGESP on WEHI231 cell spreading was also examined. Neither of these two peptides significantly inhibited WEHI231 cell spreading on the V form of FN, although the GRGDSP peptide blocked the attachment and spreading of NIH 3T3 cells on FN as expected.

Shorter peptides from within this segment were synthesized and tested for their ability to inhibit WEHI231 cell spreading (Figure 9). Whereas a 10 amino acid peptide (V10) comprising the C-terminal segment of the V25 peptide was almost as effective an inhibitor of spreading as the V25 peptide itself, two other 10 amino acid peptides, V10a and V10b, were ineffective in inhibition, as were two scrambled peptides, V10S1 and V10S2, which contained the same amino acids as V10 but in different orders (Figure 9). In these experiments, a 14 amino acid peptide, V14, which overlaps partially with V10, and GRGDSP both showed slight inhibition of spreading. These data localize the site within V25 that

is necessary for promoting cell spreading to a 10 amino acid segment.

It is known that integrin $\alpha_5\beta_1$ is a major functional FN receptor on many cells including NIH 3T3 cells (Solowska et al. 1989) and it recognizes the central binding domain of FN (Pierschbacher, M.D., and Ruoslahti, E., Nature, 309:30-33 (1984); Pierschbacher, M.D., and Ruoslahti, E., Proc. Natl. Acad. Sci. USA, 81:5985-5988 (1984); Pytela, R., et al., Cell, 40:191-198 (1985); Ruoslahti, E., and Pierschbacher, M.D., Science, 238: 491-497 (1987); Buck, C.A., and Horwitz, A.F., Annu. Rev. Cell Biol., 3:179-205 (1987); Hynes, R.O., Cell, 48: 549-554 (1987)). This can explain the similar degrees of spreading of NIH 3T3 cells on all forms of recombinant FNs since the central cell binding domain is present in all forms. On the other hand, it appears that a different FN receptor is expressed on the surface of WEHI231 cells, which can only interact with the V form of FN, through recognition of the alternatively spliced V25 segment.

The surface integrin expression on WEHI231 cells in comparison with that on NIH 3T3 cells were characterized. Cells were surface labeled with $^{125}I$, and detergent extracts were immunoprecipitated with several different antibodies. Rabbit antiserum for integrin $\beta_1$ was raised against a $\beta_1$ cytoplasmic domain peptide and reacts specifically with the $\beta_1$ subunit from many species, including mouse (Marcantonio, E.E., and Hynes, R.O., J. Cell Biol., 106:1765-1772 (1988)). The antiserum precipitated $\beta_1$ subunits (around 120 kd) together with the larger $\alpha$ subunits from both NIH 3T3 and WEHI231 cells.

Two $\alpha$-specific antisera ($\alpha_3$ and $\alpha_5$) were also raised against their cytoplasmic domain peptides (Hynes, R.O., et al., J. Cell Biol., 109: 409-420 (1989)) and cross-react with the mouse proteins (Solowska et al.(1989)). Both $\alpha_3$ and $\alpha_5$ were precipitated from the NIH 3T3 cell extract but not from WEHI231 cell extracts. The fourth antibody (rat monoclonal R1-2) was raised against the mouse integrin $\alpha_4$ subunit, which is part of the murine Peyer's patch-specific lymphocyte homing receptor (Holzmann, B., et al., Cell, 56:37-46 (1989)). Sepharose beads coupled with this antibody did not react with any proteins from NIH 3T3 cells but precipitated a major protein complex from WEHI231 cells, which comigrated with that precipitated by antibody against the integrin $\beta_1$ subunit from the same cells.

Two smaller proteins ($M_r$ 70,000 and $M_r$ 80,000) were sometimes present in immunoprecipitates of the $\alpha_4\beta_1$ complex. These are probably the degradation products of the $\alpha_4$ subunit (Hemler, M.E., et al., J. Biol. Chem., 262:11478-11485 (1987); Holtzman et al., Ibid). Perhaps because of extensive postranslational modification in WEHI231 cells, the $\alpha_4$ band could not be resolved from $\beta_1$ very well on SDS-PAGE. To confirm that the heterogeneous smear ranging from $M_r$ 120,000 to $M_r$ 150,000 was indeed composed of two noncovalently associated subunits typical of integrins, immunoprecipitations with antiserum against $\beta_1$ both before and after SDS denaturation were carried out. Only the lower part of the smear was precipitated by the antiserum after dissociation of the complex by SDS. This also suggested that the upper part was the $\alpha_4$ subunit, in agreement with its molecular weight of

150,000 (Hemler, et al., Ibid (1987); Holzmann et al., Ibid). Furthermore, the typical shift in apparent molecular weight on SDS-PAGE of the $\beta_1$ subunit after reduction was not observed, perhaps because of the heterogeneity within the band. These results indicated that major integrin complexes on NIH 3T3 cells were $\alpha_3\beta_1$ and $\alpha_5\beta_1$, while the major one on WEHI231 cells was $\alpha_4\beta_1$, and raise the possibility that $\alpha_4\beta_1$ is the cell surface receptor of WEHI231 cells responsible for their spreading on the V form of FN.

Since the V25 region is probably the active site in the V segment, this peptide was coupled to Sepharose beads and used to identify the WEHI231 cell receptor for the alternatively spliced V segment of rat FN. V25-Sepharose beads were used in a direct binding assay. WEHI231 cells were surface labeled with $^{125}I$, extracted with n-octyl glucoside, and then incubated either with mock-activated Sepharose beads or with V25-Sepharose beads in the absence or presence of various competing peptides. The bound materials were eluted with SDS and analyzed by SDS-PAGE. Cell surface proteins appearing on the gel as a heterogenous smear between 120-150 kd were shown to bind to V25-Sepharose beads specifically. The heterogenous smear in this region is reminiscent of the integrin $\alpha_4\beta_1$ immunoprecipitated from the surface of WEHI231 cells, suggesting possibly a direct interaction of integrin $\alpha_4\beta_1$ with the V25 peptide. Furthermore, this binding was effectively competed by soluble V25 peptide as well as by the V10 peptide. The V14 peptide also partially inhibited the binding (estimated at 25% by densitometry), while peptides GRGDSP or GRGESP had little

effect on the binding. The control peptides V10a, V10b, and V10s2 (see Figure 18) were also ineffective in blocking binding. These results parallel the effectiveness of these peptides in inhibiting WEHI231 cell spreading on the V form of FN (Figure 9). In these direct binding experiments, the scrambled control peptide, V10s1, inhibited partially.

Taken together, these results support the hypothesis that the V25 peptide is the active site for mediating WEHI231 cell spreading rather than inhibiting the cell spreading by interfering with a nearby site. The parallelism between the ability of various peptides to inhibit WEHI231 cell spreading and their ability to interfere with the binding of the 120-150 kd proteins to V25-Sepharose strongly suggests that the 120-150 kd proteins are the cell surface receptor mediating the spreading.

To test whether the 120-150 kd proteins are integrin complex $\alpha_4\beta_1$, V25-Sepharose column was then used to purify the receptor by affinity chromatography. WEHI231 cells were iodinated and extracted with n-octyl glucoside in buffer containing divalent cations. The extracts were loaded onto the V25-Sepharose column by incubating with the beads for 1 hour at 4°C and then washed extensively. The bound materials were eluted sequentially with buffers containing 1 mg/ml V14 peptide and 1 mg/ml V10 peptide. Aliquots of each fraction were analyzed on SDS-PAGE. Consistent with the direct binding experiments, proteins migrating as a 120-150 kd smear on the gel were bound to V25-Sepharose beads. About 40% of the bound material (as estimated by cpm) was eluted with the V14 peptide, and the remainder was eluted with the V10 peptide.

Furthermore, the same bound material could be eluted completely with either the V25 peptide or the V10 peptide when applied first. Fractions from both V14 peptide eluates (peak I) and V10 peptide eluates (peak II) were then pooled and analyzed along with the starting material by immunoprecipitation with several antibodies specific for integrin subunits. The antibodies against either the integrin $\beta_1$ or $\alpha_4$ subunits precipitated the 120-150 kd proteins from both peak I and peak II, as well as from the starting material. These results suggested that the 120-150 kd proteins bound to V25-Sepharose beads were indeed the integrin $\alpha_4\beta_1$ complex. The results indicate that integrin $\alpha_4\beta_1$ is a functional FN receptor and recognizes the alternatively spliced segment V25. This conclusion was confirmed for $\alpha_4\beta_1$ complexes isolated from murine melanoma B16-F10 cells. The results also suggest that the interaction between integrin $\alpha_4\beta_1$ and the V25 peptide occurs in the V10 peptide region but could be affected partially by the overlapping V14 peptide.

To prove that the interaction between integrin $\alpha_4\beta_1$ and the V25 peptide in the V form of FN is responsible for mediating WEHI231 cell spreading on the V form of FN, an antibody blocking experiment was carried out. WEHI231 cells were preincubated with the rat monoclonal antibody R1-2 specific for integrin $\alpha_4$, anti-IgM or rat IgG antibodies for 30 min at 4°C. The cells were then seeded in the dishes coated with saturating amounts (60 $\mu$g/ml) of the V form of FN. The percentage of cell spreading was then quantitated. At two different concentrations, R1-2 almost completely abolished the spreading of WEHI231 cells (>95% reduction). In contrast, spreading of

control samples incubated with Rat IgG was not affected significantly. Furthermore, incubation with a rat monoclonal antibody against mouse IgM, a major surface protein of WEHI231 cells (Ralph, P., Immunol. Rev. 48:107-121 (1979)), did not inhibit its spreading appreciably. This excluded the possibility that R1-2 blocked WEHI231 cell spreading simply by binding to their surfaces. Since R1-2 is a monospecific antibody for mouse integrin $\alpha_4$, these results prove that lymphoid WEHI231 cells respond to the V form of FN in vitro by their surface integrin receptor $\alpha_4\beta_1$.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

## CLAIMS

1. A method of producing a homogeneous recombinant cellular fibronectin homodimer that is biologically active, comprising introducing into an appropriate host cell a recombinant vector comprising a full length cDNA encoding cellular fibronectin of mammalian origin and maintaining cells containing the recombinant vector under conditions appropriate for expression of the cellular fibronectin.

2. A method of Claim 1, wherein the cellular fibronectin of mammalian origin is rat cellular fibronectin or human cellular fibronectin.

3. A method of producing a recombinant cellular fibronectin which is a homodimer, comprising transfecting an appropriate host cell with a recombinant retrovirus comprising full length cDNA encoding a cellular fibronectin of mammalian origin and maintaining cells transfected with the recombinant retrovirus under conditions appropriate for integration of the cDNA into host cell genomic DNA and expression of the integrated cDNA.

4. A method of Claim 3, wherein the host cell is NIH 3T3 cells or WEHI231 cells.

5. A method of Claim 4, wherein the cellular fibronectin of mammalian origin is rat cellular fibronectin or human cellular fibronectin.

6. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by the method of Claim 1.

7. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by the method of Claim 3.

8. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, produced by a method comprising introducing into an appropriate host cell a recombinant vector comprising a full length cDNA encoding cellular fibronectin of mammalian origin and maintaining cells containing the recombinant vector under conditions appropriate for expression of the cellular fibronectin.

9. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, in which region EIIIB is not present.

10. A recombinant homogeneous cellular fibronectin homodimer of mammalian origin that is biologically active, in which region EIIIA is not present.

11. A recombinant retrovirus comprising a full length cDNA encoding cellular fibronectin of mammalian origin.

12. A recombinant retrovirus of Claim 11, which further comprises a gene encoding a selectable marker.

13. A recombinant retrovirus of Claim 13, wherein the gene encodes G418 resistance.

14. A recombinant cellular fibronectin of mammalian origin in which the carboxy terminal 25 amino acids of region V are not present.

15. A recombinant cellular fibronectin of Claim 14, wherein the fibronectin is a heterodimer or homodimer.

# Fibronectin and its variants

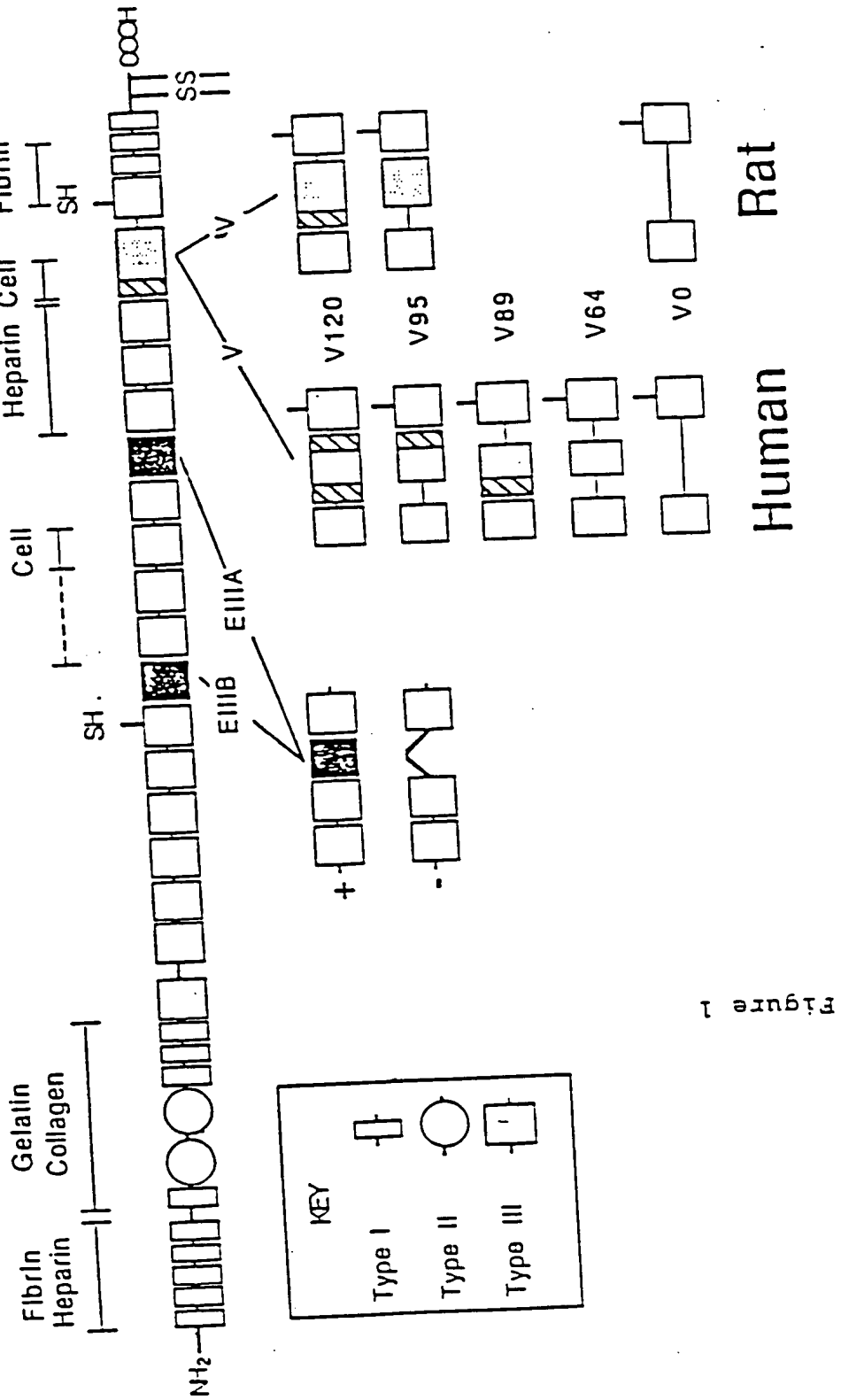


Figure 1

FIGURE 2

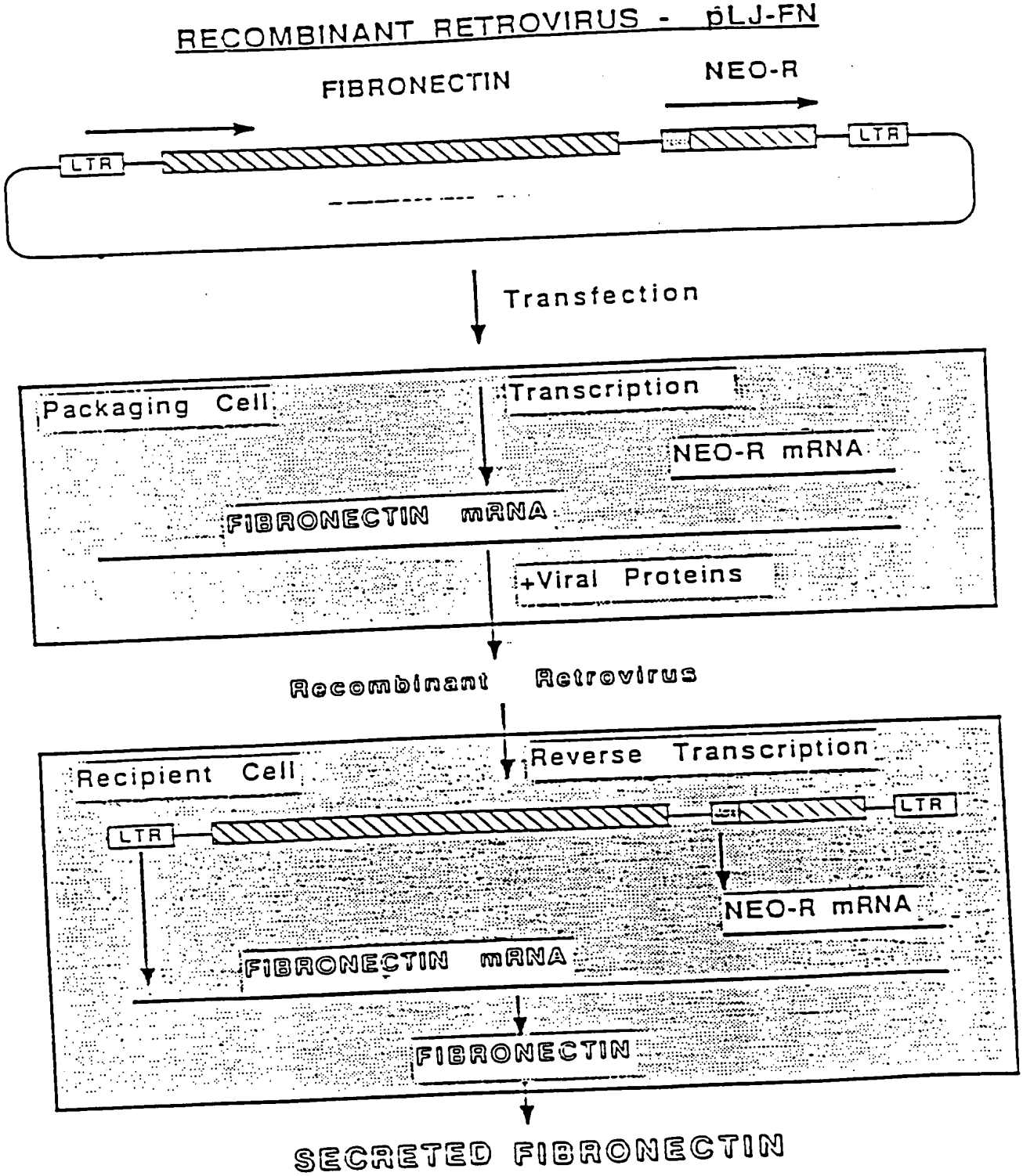
RECOMBINANT RETROVIRUS - pLJ-FN
FIBRONECTIN
NEO-R
LTR
LTR
Transfection
Packaging Cell
Transcription
NEO-R mRNA
FIBRONECTIN mRNA
+Viral Proteins
Recombinant Retrovirus
Recipient Cell
Reverse Transcription
LTR
LTR
NEO-R mRNA
FIBRONECTIN mRNA
FIBRONECTIN
SECRETED FIBRONECTIN

FIGURE 4

Secreted Recombinant Fibronectins From WEHI231 Cells

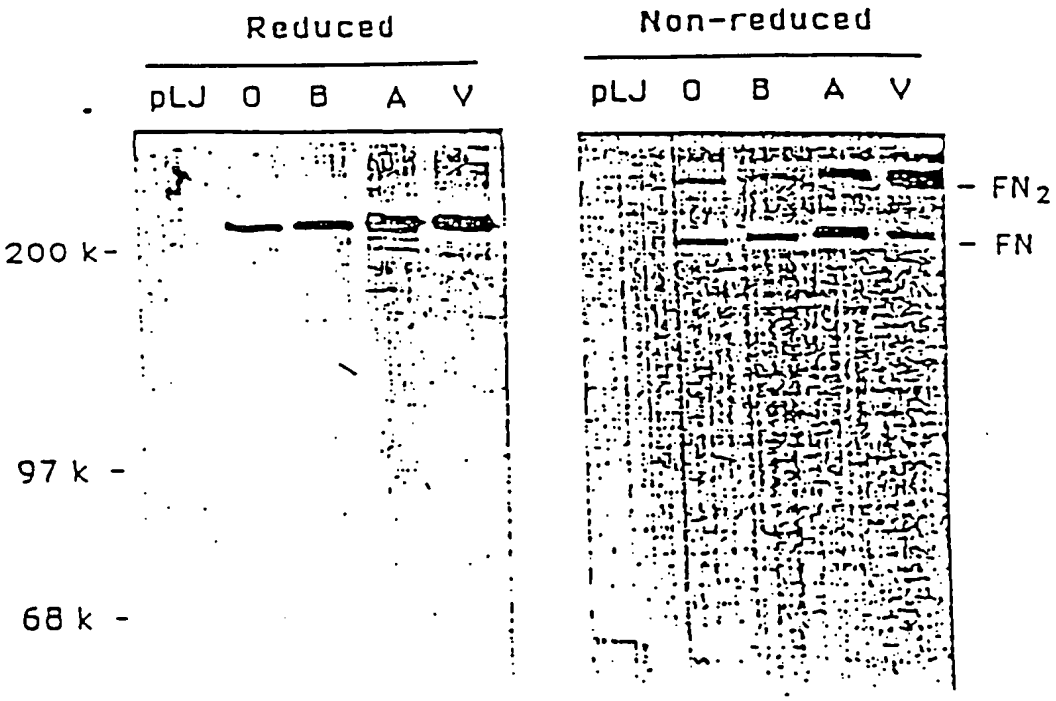

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FIGURE 3

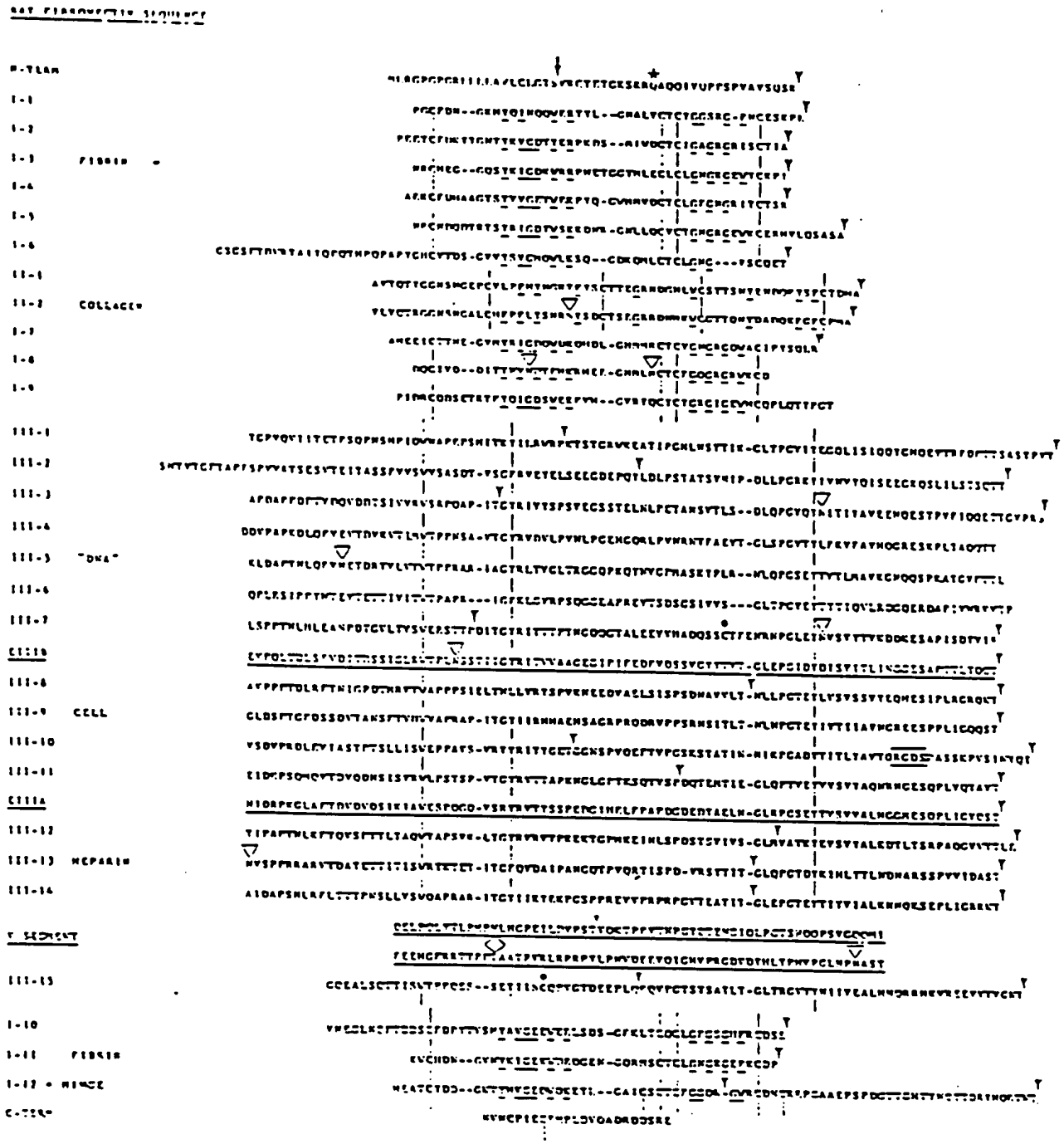


FIGURE 5

B16F10 MELANOMA CELLS SPREAD ON RECOMBINANT FN

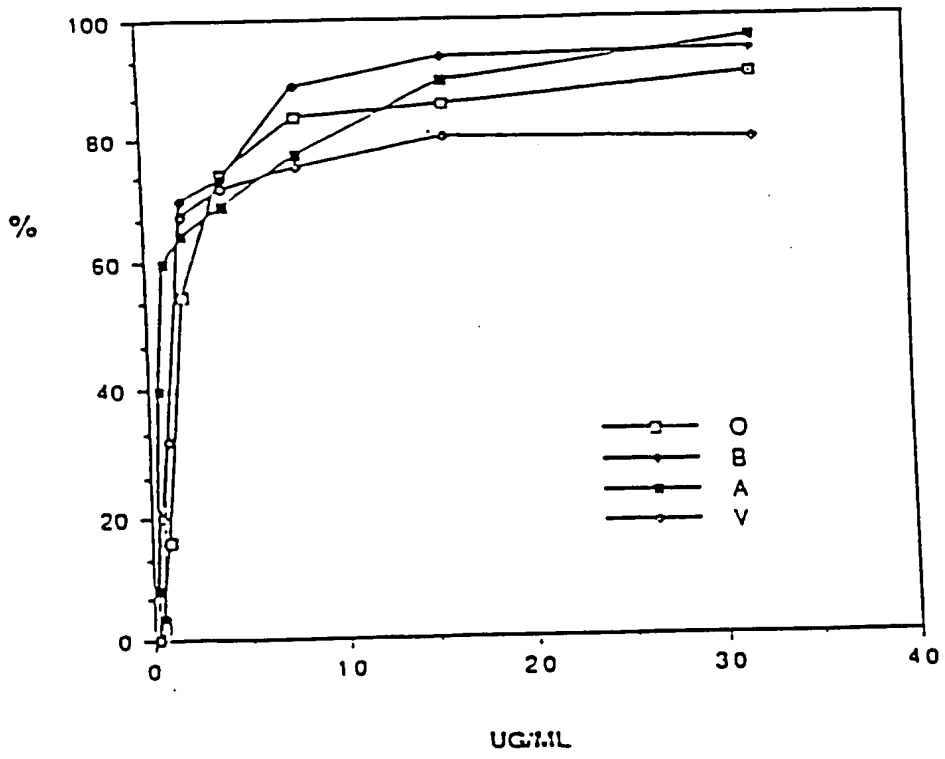


FIGURE 6

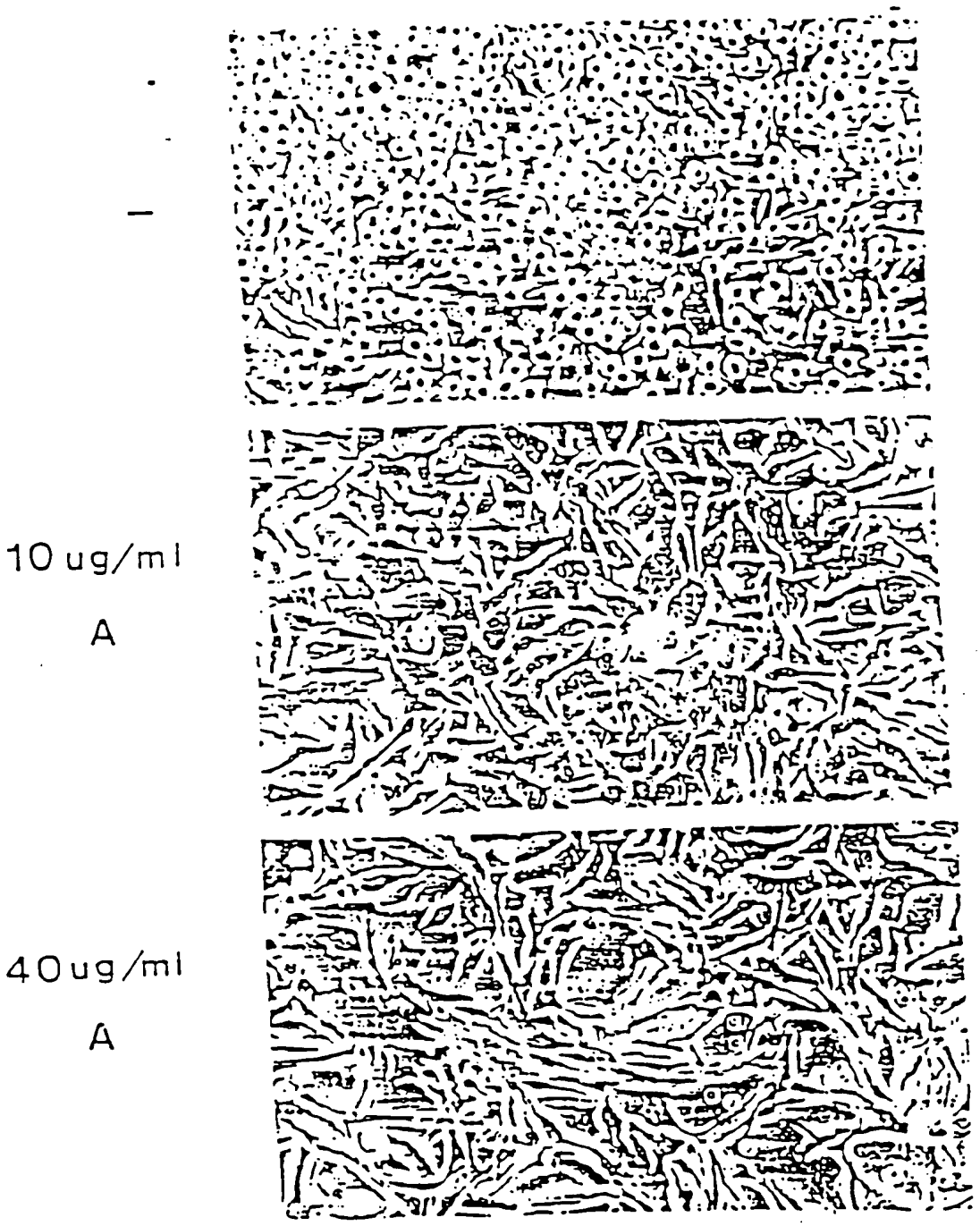
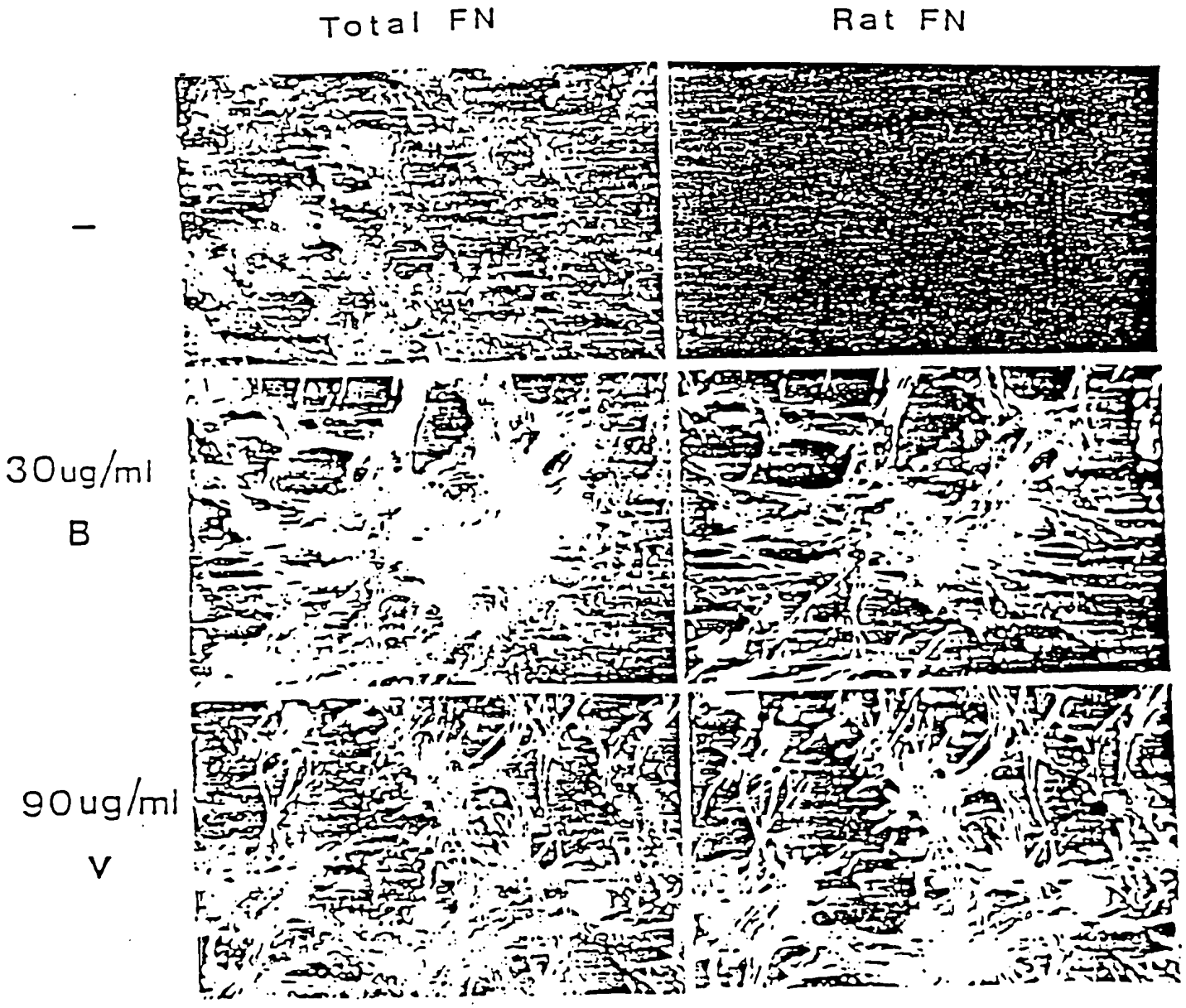


FIGURE 7



8/8

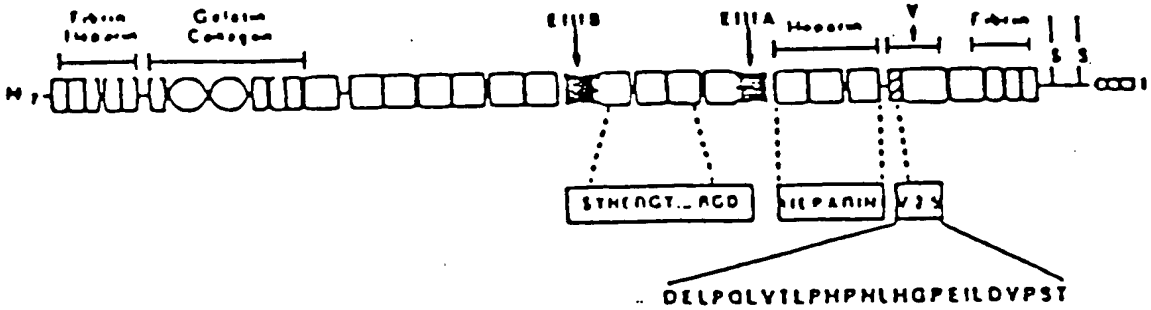



FIGURE 8

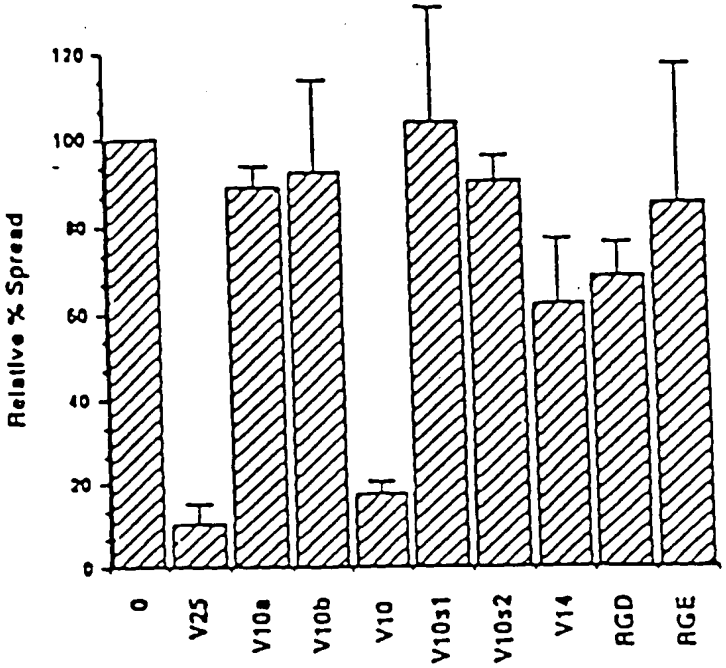



FIGURE 9A

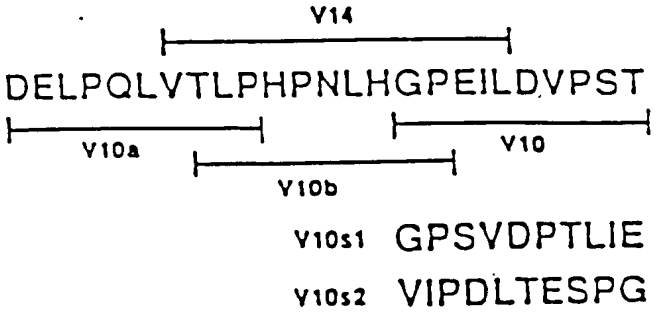



FIGURE 9B

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9000650 SA 34681

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 12/06/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| EP-A- 0207751 | 07-01-87 | AU-A- 5931586 | 08-01-87 |
| | | JP-A- 62089699 | 24-04-87 |

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00650

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 P 21/02, C 07 K 13/00, C 12 N 15/12, C 12 N 15/86

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[5] | C 12 N, C 12 P |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | EP, A, 0207751 (DELTA BIOTECHNOLOGY) 7 January 1987 see claims | 1-15 |
| Y | EMBO Journal, volume 4, no. 7, 1985, IRL Press Limited, (Oxford, GB), A.R. Kornblihtt et al.: "Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single single gene", see pages 1755-1759 | 1-15 |
| Y | EMBO Journal, volume 6, no. 9, 1987, IRL Press Limited, (Oxford, GB), J.E. Schwarzbauer et al.: "Multiple sites of alternative splicing of the rat fibronectin gene transcript", pages 2573-2580 see page 2576, column 2, lines 3-7 | 1-15 |

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 4th May 1990 | 13. 05. 90 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | M. PEIS |

Form PCT/ISA/210 (second sheet) (January 1985)